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17182. Stimulation of Gastric Secretion in Man by Theophylline Ethylenediamine.

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Roth and Ivy[1-3] reported that caffeine parenterally or intragastrically, stimulates gastric secretion in cat and man. They noted no effect when caffeine was given alone in the dog. Robertson and Ivy[4] observed that caffeine and theophylline potentiate the effect of histamine on gastric secretion in dogs, but did not stimulate when given alone. Wood[5] noted that in some cats theophylline and theobromine alone stimulated gastric secretion. No reference to the effect of theophylline on gastric secretion in man could be found in the literature, and so the present study was undertaken.

Method. After a 12 hour fast, a Levine tube was introduced into the stomach and the contents aspirated at 15 minute intervals. After the fourth sample was withdrawn, 0.5 g of theophylline ethylenediamine was administered slowly intravenously dissolved in 10 cc of physiological saline, or through the stomach tube in 200 cc of water. The gastric contents were aspirated at 15 minute intervals thereafter for 90 minutes except that with the orally administered drug the first period after introduction of the drug into the stomach was 20 minutes in length instead of 15 minutes. For each sample the total milligrams of free acid was determined. Ten subjects received the theophylline intravenously and ten received the theophylline through the stomach tube.

Results and comment. The results obtained with intravenous administration are shown in Fig. 1, with oral administration in Fig. 2. The maximum effect of theophylline is noted 15 minutes after intravenous administration, whereas with the oral preparation the maximum effect occurred 45 minutes after administration.

These results indicate that theophylline is

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[1] Roth, J. A., and Ivy, A. C., *Am. J. Physiol.*, 1944, **141**, 454.

[2] Roth, J. A., and Ivy, A. C., *Gastroenterology*, 1944, **2**, 274.

[3] Roth, J. A., and Ivy, A. C., *Am. J. Physiol.*, 1944, **142**, 107.

[4] Robertson, C., and Ivy, A. C., *Fed. Proc.*, 1949, **8**, 133.

[5] Wood, D. R., *British Med. J.*, 1948, **2**, 283.

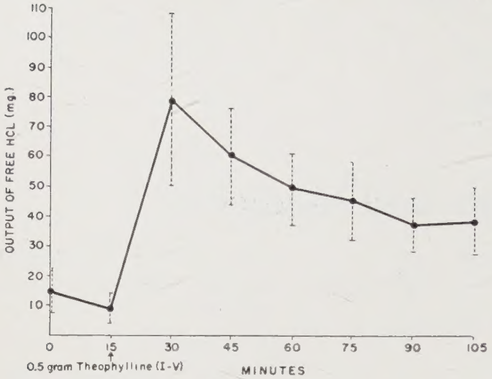

FIG. 1.
Stimulation of free HCl output by intravenous administration of theophylline ethylenediamine. Each dot represents the mean value of determinations on 10 subjects. The vertical bars represent the standard errors of the respective means.

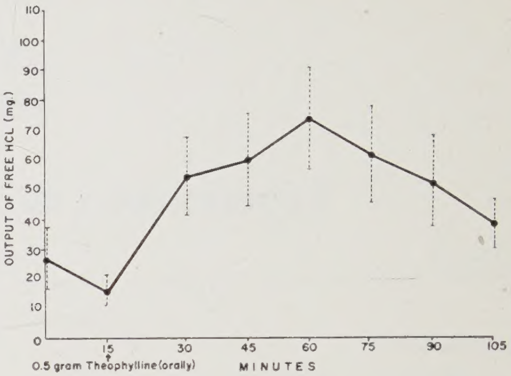

FIG. 2.
Stimulation of free HCl output by oral administration of theophylline ethylenediamine. See legend of Fig. 1.

similar to caffeine in its effect on gastric secretion in man.

In the course of the study 3 patients received a tablet form of theophylline with variable response, and 4 patients received theobromine orally with no gastric stimulation.

No decision can be made as to whether the stimulation produced by orally administered aminophylline is due to direct local action or to an action after absorption.

Summary. Theophylline stimulates the secretion of acid gastric juice in man on oral or intravenous administration.

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17183. Bacterial Conversion of Dextrin into a Polysaccharide with the Serological Properties of Dextran.

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The purpose of the present paper is to report the capacity of certain acetic acid bacteria, and of cell-free solutions of enzymes obtained from them, to synthesize from dextrin material with serological properties like those which we have earlier described for dextran.[1] Hitherto, the only enzymatic pathway known for the synthesis of dextran was that from sucrose.[2,3] We know of no report of interconversion of material of the starch-glycogen class and dextran, although both possess related chemical structures and can indeed be synthesized from the same substrate (sucrose) by appropriate bacterial enzyme systems.[2-5] Ultimate proof of the biological synthesis of dextran from dextrin must of course rest upon chemical studies, which are now in progress. However, the unequivocal nature of the present serological data leaves little doubt that such a conversion does in fact occur.

British brewing bacteriologists[6-11] have for some years recognized that certain varieties of acetic acid bacteria, now known as *Acetobacter viscosum* and *Acetobacter capsulatum*, often are associated with the type of spoilage of beer known as "ropiness." Recently Shimwell[12,13] has shown that the production of ropiness by these bacteria depends upon their capacity to form slime from dextrin, a natural constituent of beer. This author found that cultures of *A. viscosum* and of *A. capsulatum* became grossly viscous in dextrin-rich beer or in a medium of yeast extract containing 4 per cent dextrin, but not in beer devoid of dextrin or in yeast extract media in which the dextrin was omitted or replaced by glucose, fructose, maltose, or sucrose. It is of historical interest that Henneberg[14] fifty years previously had reported slime formation in dextrin-rich media (also often in beer) as a differential feature of the related *Bacterium* (*Acetobacter*) *industrium*.

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[1] Sugg, J. Y., and Hehre, E. J., *J. Immunol.*, 1942, **43**, 119.

[2] Hehre, E. J., and Sugg, J. Y., *J. Exp. Med.*, 1942, **75**, 339.

[3] Hehre, E. J., *J. Biol. Chem.*, 1946, **163**, 221.

[4] Hehre, E. J., and Hamilton, D. M., *J. Bact.*, 1948, **55**, 197.

[5] Hehre, E. J., *J. Biol. Chem.*, 1949, **177**, 267.

Little has been recorded of the nature of the slimy material produced by the acetic acid bacteria associated with ropy beer. Hampshire[8] isolated a mucilaginous material from beer made ropy by inoculation with *Acetobacter* R, a variety now said to be closely related to *A. viscosum*.[13] This material was ash free, contained approximately 1% nitrogen, and on acid hydrolysis yielded 89.5% reducing sugar, calculated as glucose. Polarimetric readings were stated not to agree with this figure, but nevertheless the author concluded that the material was "of the nature of a dextran", *i.e.*, a polyglucoside. In the case of the slimy materials known to have been produced from dextrin by *A. viscosum* and *A. capsulatum* we were unable to find any published information apart from the statement of Shimwell[12] that these must be of carbohydrate nature because of their origin.

The present study was made with two strains of *Acetobacter*, procured from the National Collection of Type Cultures, capable of producing a gelatinous type of growth in media containing dextrin: *i.e.*, *A. viscosum* 7216, isolated from a ropy top fermentation of beer by Tosic, and *A. capsulatum* 4943, isolated from ropy beer by Shimwell.[9,12] The capacity of these strains to produce material serologically like dextran was established by experiments made in comparison with a strain (B) of *Leuconostoc mesenteroides* originally used in the establishment of the serological reactivity of sucrose-derived dextrans.[1]

The utilization of dextrin as the substrate for the formation of serologically reactive material by the *Acetobacter* cultures, in contrast to the utilization of sucrose for the synthesis of serologically similar material by the *Leuconostoc mesenteroides* can best be illustrated by the following experiment. The *Acetobacter* and *Leuconostoc* strains, after preliminary passage in glucose broth, were inoculated into a series of tubes of broth consisting of 0.5% Difco yeast extract plus 5% of one of a number of different carbohydrates, including dextrin and sucrose. The dextrin was of bacteriological grade, that is, a starch-free, alcohol-precipitated product; the sucrose was a sample of beet sugar known to be free of the traces of material precipitating with dextran-reactive antiserums which occur in most lots of reagent and commercial sucrose;[15,16] all the other sugars were of reagent grade. After incubation at 25°C for one week, the cultures which had grown were centrifuged, and their neutralized supernatant fluids tested at several dilutions for capacity to give precipitation with each of two different dextran-

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[6] Baker, J. L., Day, F. E., and Hulton, H. F. E., *J. Inst. Brewing*, 1912, **18**, 651.

[7] Day, F. E., and Baker, J. L., *Cent. f. Bakt., Abt. 2*, 1913, **36**, 433.

[8] Hampshire, P., *Bull. Bureau of Bio-Technology*, 1922, **1**, 179, 199.

[9] Shimwell, J. L., *J. Inst. Brewing*, 1936, **42**, 585.

[10] Comrie, A. D., *J. Inst. Brewing*, 1939, **45**, 342.

[11] Walker, T. K., and Tosic, J., *J. Inst. Brewing*, 1945, **51**, 245.

[12] Shimwell, J. L., *J. Inst. Brewing*, 1947, **53**, 280.

[13] Shimwell, J. L., *Wallerstein Labs. Commun.*, 1948, **11**, 27.

[14] Henneberg, W., *Cent. f. Bakt., Abt. 2*, 1898, **4**, 933 (Abstract).

[15] Neill, J. M., Hehre, E. J., Sugg, J. Y., and Jaffe, E., *J. Exp. Med.*, 1939, **70**, 427.

[16] Neill, J. M., Sugg, J. Y., Hehre, E. J., and Jaffe, E., *Am. J. Hyg.*, 1941, **34B**, 65.

TABLE I.

Capacities to Precipitate with Dextran-reactive Antiserums Shown by the Culture Fluids of *Acetobacter* and *Leuconostoc* Grown with Different Carbohydrates.

Strain	Highest dilution of the fluids from cultures with different carbohydrates that gave precipitation with type 2 and with type 20 pneumococcus antiserums*				
	Dextrin	Maltose	Sucrose	Raffinose	Other sugars†
Acetobacter viscosum, 7216	50,000	10‡	0	0	0
ʼʼ *capsulatum,* 4943	50,000	0‡	0	0	0
Leuconostoc mesenteroides, B	0	0	100,000	20	0

* 0 = no precipitation with either antiserum in tests with 1:10 or 1:100 dilutions of the culture fluids.

† Glucose, fructose, mannose, galactose, sorbose, xylose, arabinose, mannitol, inositol, trehalose, α-methylglucoside, lactose, melibose, or inulin.

‡ Fluids from cultures grown with a different sample of commercial ʻʻC. P.ʼʼ maltose gave reactions at dilutions as high as 1:400 with the dextran-reactive antiserums.

reactive antiserums; these were a type 2 pneumococcus antiserum (1:50 dilution) and a type 20 pneumococcus antiserum (1:18 dilution) known to give precipitation with purified *Leuconostoc mesenteroides* strain B dextran in dilutions as high as 1:4 million. Mixtures of 0.2 ml of diluted culture fluid and 0.2 ml of diluted antiserum in clear 10 x 75 mm test tubes were incubated at 37°C for 1 hour and then observed for the development of cloudiness or precipitation.

As shown in Table I, large amounts of material reactive with the type 2 and the type 20 pneumococcus antiserums were present in the fluids of the *A. viscosum* and *A. capsulatum* cultures grown with dextrin, while with one exception, none was detected in the fluids of the cultures grown with other carbohydrates. Traces of reactive material were found in the fluids of the cultures with maltose, but we believe these can be accounted for by the presence of minute amounts of dextrin in the maltose. A large amount of material (dextran) reactive with the same antiserums was produced from sucrose by the *Leuconostoc* culture, and a trace also from raffinose, but none was produced from the dextrin or from any of the other sugars tested.

More complete information on the nature of the serologically reactive substance produced by *A. capsulatum* 4943 has been obtained by studies made with material isolated from a dextrin broth culture. Several liters of viscous culture fluid yielded an amount (approximately 15 g) of serologically reactive polysaccharide which corresponded to more than 20% of the weight of the dextrin used in the medium. An account of the separation, purification, and chemical properties of the *A. capsulatum* polysaccharide will be published in a subsequent report. Results of the serological precipitation tests which we have made with this material in comparison with purified dextran isolated from a sucrose broth culture of *Leuconostoc mesenteroides* B[1] are shown in Table II.

Both the *A. capsulatum* polysaccharide and the *L. mesenteroides* dextran gave visible precipitation with type 2 and type 20 pneumococcus antiserums in dilutions from 1:10,000 to 1:4 million; both gave precipitation also with type 12 pneumococcus antiserum though only at much lower dilutions; and both failed to react at 1:10,000 or higher dilutions with any of the control serums, which included high-titered antipneumococcal serums of several types, normal serums from rabbits which furnished type 2 and 20 serums, and an antiserum capable of precipitating with high dilutions of the polyfructoside levan. The mutual cross-reactions with the types 2, 20 and 12 pneumococcus antiserums, given by the *A. capsulatum* 4943 and *L. mesenteroides* B polysaccharides, and especially the comparably high degree of reactivity versus the types 2 and 20 in comparison to the lower degree of reactivity versus the type 12 are evidences of an extraordinarily close serological likeness between these two materials. Additional evidence for the close serological similarity, not presented in Table II, was that absorption of type 2 pneumococcus anti-

TABLE II.
Serological Similarity of the Dextrin-derived Polysaccharide of *Acetobacter capsulatum* to the Sucrose-derived Dextran of *Leuconostoc mesenteroides.*

Kind of serum	Dilution of serum	Highest dilution of antigen that gave precipitation*	
		Acetobacter capsulatum polysaccharide	*Leuconostoc mesenteroides* dextran
Antipneumococcus, type 2 (Led)†	1:50	4,000,000	4,000,000
” ” 2 (rabbit S5)	1:25	2,000,000	2,000,000
” ” 20 (” 02)	”	4,000,000	4,000,000
” ” 12	”	50,000	10,000
” ” 1, 3, 5, 8, 14 (Led)†	1:25, 1:50	0	0
Normal, pre-immunization (rabbits S5, 02)	1:12, 1:25	0	0
Levan-reactive, *Bacillus* N9	1:12	0	0

* 0 = no precipitation with 1:10,000 or any higher dilution of the polysaccharide.

† Refined, or concentrated, rabbit antiserums kindly supplied by Dr. H. D. Piersma, Lederle Laboratories, Pearl River, N.Y.

serum with either polysaccharide removed the antibodies reactive with both polysaccharides.

The enzyme system in *A. capsulatum* responsible for the conversion of dextrin into material serologically like dextran is readily obtainable in solution, free from bacterial cells. Using the fluids from glucose broth cultures as starting material, we have been able to prepare potent concentrates by a method previously described.[5] When "bacteriological" dextrin, Lintner's soluble starch, or certain hydrolysates made either from amylopectin *or from crystalline amylose* are incubated with such enzyme preparations, amylase-resistant polysaccharide material that gives serological precipitation with dextran-reactive antiserums is formed. These results suggest that the action of the *Acetobacter* enzyme is to transfer 1:4 linked glucose units to new polysaccharide material in which the units are linked in some other fashion, very likely 1:6. Although proof of the exact mechanism of the reaction must await the results of systematic chemical study, recognition of the enzymatic convertibility of amylaceous to non-amylaceous polysaccharide by itself is of biological significance.

Summary. Cultures of certain acetic acid bacteria from "ropy" beer, when grown with dextrin but not when grown with other common carbohydrates, produce abundant amounts of polysaccharide material which has serological properties like those of the dextran which is produced from sucrose by *Leuconostoc mesenteroides*. A cell-free enzyme system, capable of causing the conversion of dextrin to material serologically like dextran, has been obtained from one of the cultures.

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17184. Anoxic Diversion of the Renal Cortical Blood Flow.

K. J. FRANKLIN, L. E. MCGEE, AND E. ULLMANN. (Introduced by J. F. Fulton.)
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During 1947-8 Trueta, Franklin, and Amoroso laid plans for the joint solution of various renal problems, and the research to be described, though in fact of more fortuitous origin, is to be regarded as part of that cooperative effort. Briefly, it was thought probable that the renal cortical blood flow is diverted whenever more important, or temporarily more important, organs are rendered too anoxic. Experiments were, therefore, begun with a view to testing this hypothesis, and in those to be described the whole body was subjected to anoxia in the expectation that some parts would be more sensitive than the renal cortex, and that in consequence the latter would be, reflexly or otherwise, deprived of some or all of its blood supply, the portion of the total circulating blood volume so diverted being added to the quotas allotted to more needy organs. At the time the work of Toth[1] was not known to the writers; it will be referred to below in the discussion of results.

Methods. The animals mainly used were rabbits, anaesthetized by intravenous injection of Nembutal solution, followed by administration of open ether as required; in earlier work Trueta *et al.*[2] found that anaesthesia, maintained by such means for several hours, produced no appreciable change in the renal blood flow. To exclude specific peculiarities, some tests were also made on the cat, dog, rat, and mouse.

Anoxia was produced (1) by occlusion of a straight glass cannula tied into the trachea, and (2) by administration of a lethal dose of carbon monoxide (mixture of air and coal gas).

The renal effects were observed either with both kidneys made visible by laparotomy, or with one or both kidneys exteriorized. The left kidney was exteriorized by bringing it out between the lumbar and abdominal musculatures, the right as seemed best according to the individual circumstances. To determine if the renal effects were nervously intermediated, the left kidney was denervated (usually by division of the nerve or nerves accompanying the renal artery, but on occasion by splanchnic section), and the right kidney was left intact. When desired, the state of the renal blood distribution was precisely ascertained by injecting warmed India ink in a proximal direction through a cannula tied into the distal aorta, the mesenteric artery having first been ligated. The injection was made at a pressure just above that in the aorta, and ligatures previously passed round the renal vessels were tied while the injection was at its height. Thereafter, both kidneys were exsected and placed in fixative for a day or so before being cut open and prepared for subsequent histological section.

In a few cases a 16 mm color film record was made of the anoxia production and its results. Details of the published films are given after the references at the end of this paper.

Results. Occlusion of the tracheal cannula did not usually cause an immediate cessation of respiratory efforts; indeed, these might, for a time at least, increase in rate and/or intensity. Within 20 to 60 seconds, as a rule, the occlusion produced first a darkening, then patchy paling, and finally generalized paling (sometimes spectacular in its degree) of the kidney surface, together with diminution in size of the organ and wrinkling, often very marked, of its capsule. The diminution in volume, if one can judge from cubing linear measurements, was of the order of one-sixth; an oncometer was not used as it would have introduced complicating factors. Sections of innervated kidneys removed at the height of the anoxia effects showed anaemia of the cortical portions; in the denervated kidneys there was no such diversion of cortical blood flow, though it was possible to produce it in such organs by intravascular injection of adrenaline. In some of the former cases there was more blood than normal in the medulla; in others less. When re-entry of air into the lungs was allowed to occur, blood rapidly returned to the kidney surface, at first discretely, but almost immediately thereafter all over. In their rapidity, therefore, the renal changes after anoxia were more dramatic than those produced during its development. In contrast to the restoration of color was the disappearance of the surface wrinkling, which often took a minute or two for its completion. The whole tracheal occlusion experiment could be repeated at very short intervals and, at least in the case of the exteriorized kidneys, over a total period of several hours. Administration of carbon monoxide, in the dosage employed, produced equally marked diversion of blood from the cortex but the change was irreversible. Two final notes may be added to the above account. In the first place, partial denervation of one kidney, which was effected in a few cases, divided the single organ into a part which responded to anoxia and a part which did not; this was a convenience for some purposes. Secondly, if the stomach was over-full or the uterus seriously enlarged as a result of pregnancy, the kidneys tended to be found partially "shunted," if one may use the American expression for diversion of the cortical blood supply, from the outset.

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[1] Toth, L. A., *Am. J. Physiol.*, 1940, **129**, 532.

[2] Trueta, J., Barclay, A. E., Daniel, P. M., Franklin, K. J., and Prichard, M. M. L., Studies of the renal circulation, 1947; Franklin, K. J., Anoxic diversion of the renal cortical blood flow, Part I, 1948; Ullmann, E., McGee, L. E., and Franklin, K. J., Anoxic diversion of the renal cortical blood flow, Part II, 1949. Oxford: Blackwell Scientific Publications, Ltd.

Discussion. Toth's[1] anoxia experiments were carried out in dogs, and paling of the surface of the dog's kidney during diversion of the cortical blood flow is not readily detected because (1) the capsule is relatively thick, and (2) the cortical color changes are masked by superficial vessels which are unconcerned, or little concerned, in the diversion. Further, the findings of Trueta *et al.*, which showed the significance of such cortical diversion as opposed to an overall reduction in renal arterial inflow, were not begun until 1945. In a future paper two of the present writers (L.E.M, E.U.) will give quantitative details about the degrees of anoxia and of hypercapnia which are required for the production of the renal circulatory changes, and will describe the fluctuations of arterial blood pressure recorded during these changes, together with other features of interest. They may also give evidence about the urine flow as affected by anoxia, and offer a possible explanation for the time-lag between the return of color to the kidney surface and the disappearance of the wrinkling from that surface. In this preliminary account, however, the main object is to describe the more obvious features and to point out that the simultaneous use of innervated and denervated kidneys, with the completeness of the denervation shown by a tracheal occlusion test, allows one rapidly to determine if a poison, such as dioxan (see De Navasquez,[3]) or a bacterial toxin, affects the kidneys through nervous intermediation or otherwise. Publication of this present paper is, therefore, a necessary prelude not only to the communication of further finding about anoxia effects, etc., but also to the description of the results of experiments with a number of nephrotoxic substances.

Summary. 1. Acute anoxia, sufficient in degree, produces a marked diversion of the renal cortical blood flow in the innervated, but not in the denervated, kidney.

2. Simultaneous study of the reactions of the innervated and denervated kidneys is a simple way of distinguishing the mechanisms of action of different nephrotoxic substances.

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[3] De Navasquez, S., *J. Hyg.*, 1936, **35**, 540; De Navasquez, S., *J. Path. Bact.*, 1938, **46**, 47.

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17185. Effect of Elevated Body Temperatures on Cryptococcosis in Mice.

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In a study[1] of the effects of temperature on the reproduction and viability of *Cryptococcus neoformans,* the number of viable cells decreased by 85% within 5 days at 103° F (39.4°C), but increased regularly at 99°F (37.3°C). The writer postulated that differences in body temperatures of laboratory mice and rabbits might account for differences in susceptibility to cryptococcosis. Mice, with rectal temperatures averaging 99.1°F, died

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[1] Kuhn, L. R., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 573.

TABLE I.
Survival of Mice Injected with *C. neoformans* and Maintained Either at 35 to 36°C or 24 to 27°C.

Dosage of *C. neoformans*	Method of inj. and No. injected	Time after injection	No. surviving in mice maintained at temperatures indicated		
			24-27°C	35-36°C	35-36°C after being kept at 24-27°C for time indicated
					2 days
2 to 2.5 million in 0.5 ml of saline	Intravenous 15 in each temp. group	0 hr	15	15	15
		48 ”	15	11	15
		11 days	0	11	10
		20 ”	0	10	6
		30 ”	0	6	3
					8 days
Same	Intraperitoneal 18 in each temp. group	0 hr	18	18	18
		44 days	0	12	9
		60 ”	0	7	4
					2 days
120 to 150 thousand in 0.03 ml of saline	Intracerebral 25 in each temp. group	0 hr	25	25	25
		24 ”	25	21	25
		10 days	4	15	20
		20 ”	0	8	6
		30 ”	0	6	2

regularly and relatively rapidly of experimental infections, while rabbits, with a mean temperature of 103.15°F, were affected only rarely and slowly.

To determine if mice could be maintained with elevated body temperatures long enough to study their reaction to cryptococcosis, groups of Swiss mice were kept at various temperatures above 30°C. In still air at 35 to 37°C with 25 to 35% relative humidity, rectal temperatures rose rapidly and remained above 102°F.[*] Although a few mice died during an initial day or two of discomfort for all, the majority survived and were in apparent good health at the end of a month, although averaging only 1.3 g gain in weight compared with 7.1 g in room temperature controls. At this time, the rectal temperatures of 2 separate lots of heat-adapted mice averaged 102.8 and 103.4°F, ranging from 101 to 105°F.

Infections with a pathogenic strain[†] of *C. neoformans* were compared in Swiss mice weighing 17 to 19 g and kept either at 24 to 27°C or at 35 to 36°C. Groups maintained at each temperature level were injected intravenously, intraperitoneally or intracerebrally with 60-hour Sabouraud agar cultures of *C. neoformans* suspended in 0.85% sodium chloride. Doses for each group as well as the effect of environmental temperatures on survival are shown in Table I. Mice in all groups kept at 35 to 36°C outlived all those maintained at 24 to 27°C. Thus, although all of 15 intravenously injected mice kept at room temperature died of cryptococcosis within 11 days after being infected, only 4 of 15 died during the same period in the higher temperature group. Since these 4 died within 48 hours after injecting and incubating, their deaths cannot be attributed to the slowly developing cryptococcus infection. No other heat-adapted mice died until 19 days after injection. Six survived 30 days without apparent effects.

Although intraperitoneal infections developed more slowly, only 6 of 18 mice kept at 35 to 36°C died during the 44 days following injection. All of the control mice died during the period. In 3 of the 6 heat-adapted mice which died, no cryptococci were found by smear or culture. Seven were still living in apparent health 60 days after being injected.

Intracerebrally injected mice showed less striking differences between the temperature groups. Some kept at 35 to 36°C outlived all the room temperature controls, however.

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[*] Rectal temperatures were taken with narrow bulb thermometers made by Rascher and Betzold, Inc., Chicago, Ill.

[†] Army Medical School strain D-2, isolated from a human case at autopsy.

With each of the series described above, an equal number of mice was similarly injected but kept at 24 to 27°C for 2 or 8 days prior to being moved to the higher temperature. Mice in each of these sets lived longer than those kept only at room temperature, but fewer survived than in the groups placed at 35 to 36°C immediately after injecting (Table I).

Fatal infections in many of the heat-adapted mice may have accompanied body temperatures under the average of 103°F, where cryptococci remain viable or even increase *in vitro*. Also, the temperature of the incubator fell to 33 and 32°C during two overnight periods, and all rectal temperatures taken in the morning were at or below 100.5°F. Rectal temperatures of incubated mice showing signs of infection usually were below 100.5°F and decreased as the animals became moribund.

In the light of the previous *in vitro* studies,[1] the results given above suggest that a direct effect of temperature on the growth and viability of *C. neoformans* should be considered in explaining the degree of natural resistance shown by rabbits.

Inhibition of virus infections in small animals kept at high environmental temperatures has been observed, according to Francis.[2] Coplin and Mills[3] found that white mice which had been adapted to moist heat were *more* susceptible to *Streptococcus hemolyticus* than were mice kept at lower temperatures.

Temperatures of 103°F and above may be inimical to the growth and viability of other fungi causing systemic mycoses. Textbooks of medical mycology direct that cultures be incubated at both room temperature and at 37° for Monilia, Coccidioides, Histoplasma, Sporotrichum, and Blastomyces as well as for *C. neoformans*. The writer found[1] that the optimum for rapid growth of *C. neoformans* was approximately 29°C and some workers in this field now consider 28 to 30°C to be optimal for all the pathogenic fungi listed above. It would not be surprising, therefore, if more of these organisms fail to maintain themselves at elevated body temperatures.

It is interesting to consider the possible use of fever therapy, alone or combined with chemotherapy, in treating human cryptococcosis (torulosis). Specific therapy appears to be questionable or even lacking entirely,[4,5] and human cases usually show little or no elevation of body temperature. Cox and Tolhurst[6] discuss the possibilities of hyperthermia in the light of the earlier *in vitro* studies of the writer.

Summary. 1. Many mice infected with a pathogenic strain of *Cryptococcus neoformans* and maintained, with elevated body temperatures, at 35 to 36°C, lived longer than all infected mice kept at 24 to 27°C.

2. These and previous *in vitro* studies suggest that a direct effect of temperature should be considered in explaining the degree of natural resistance of rabbits to cryptococcosis.

3. Elevated body temperatures might be useful in assisting treatment of human cryptococcosis.

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[2] Francis, Thomas, Jr., In Bacterial and Mycotic Infections of Man. Edited by Dubos, R. J., 1948, p. 98, J. B. Lippincott Co.

[3] Coplin, J. W., and Mills, C. A., *Science,* 1939, **90**, 275.

[4] Cox, Leonard B., and Tolhurst, Jean C., Human Torulosis, 1946, pp. 79, 129, Melbourne University Press.

[5] Conant, Norman F., In Bacterial and Mycotic Infections of Man. Edited by Dubos, R. J., 1948, p. 600, J. B. Lippincott Co.

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17186. A Virus Isolated from Patients Diagnosed as Non-Paralytic Poliomyelitis or Aseptic Meningitis.*

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It is the purpose of this report to describe the isolation of a filtrable agent from patients with an illness resembling non-paralytic poliomyelitis which occurred during 1948 in southern New England. The virus was sought following the report by Dalldorf and Sickles,[1] and is apparently similar to that which they obtained from the feces of 2 patients diagnosed as paralytic poliomyelitis. Like their agent, the virus infected newborn albino mice and produced in them weakness and paralysis accompanied by diffuse myositis.

The following aspects of work with this virus will be described:

(1) recovery of the virus from the feces of patients and its neutralization by their sera;
(2) description of the experimental disease and some properties of the virus;
(3) inapparent infection of chimpanzees;
(4) accidental infection of man;
(5) occurrence of the virus in "poliomyelitis" patients in Ohio and North Carolina as well as in New England;
(6) occurrence of the virus in extra-human sources (sewage and flies);
(7) immunological types of the virus.

(1) *Isolation of virus from patients diagnosed as having non-paralytic poliomyelitis or aseptic meningitis.* During the summer and fall of 1948 samples of feces were collected from 16 representative patients from Connecticut and Rhode Island, 13 of whom presented clinical features consistent with non-paralytic poliomyelitis (Table I), and 3 of whom had definite muscle weakness and were diagnosed as paralytic poliomyelitis. The fecal samples from these 16 patients were tested in monkeys for poliomyelitis virus and were also examined for the agent infectious for newborn mice.

Fecal samples from 5 (Nos. 1-5) of the 13 patients with non-paralytic illnesses produced disease in newborn mice, but not in monkeys. From one to 4 rhesus monkeys (*Macaca mulatta*) were used to test each of these samples for poliomyelitis virus with negative results. Furthermore the strains recovered in newborn mice were tested in 8 additional monkeys and again the results were negative. The new virus was also found in the feces of 2 patients (No. 18-19) diagnosed as "fever of unknown origin". Lumbar puncture was done in only one of the latter patients and there was no pleocytosis.

The new virus has not been recovered by blind passage of brains of normal newborn mice, nor from separate fecal samples of 29 patients in New Haven with various other infectious and non-infectious diseases.

Fecal samples from two of the patients with non-paralytic disease (Nos. 11-12) yielded poliomyelitis virus when inoculated into monkeys, but did not produce disease in newborn mice. The 2 strains of poliomyelitis virus were typical; they were pathogenic for monkeys but not for cotton rats or mice (3 week old as well as newborn). Samples of feces from the 3 patients with clinical paralytic poliomyelitis (Nos. 13-15) failed to yield an agent when tested in either monkeys or newborn mice.

Tests for neutralization of the new virus with acute and convalescent sera of the patients revealed that:—(i) the sera of 7 patients (Nos. 1-5, 18,19) from whom the new agent was isolated, neutralized the virus (neutralization index from 1,000 to 10,000) in the convalescent stage and to a lesser degree in the acute stage; (ii) the convalescent sera from 5 other patients (Nos. 6-10) with non-paralytic illnesses also neutralized the virus;

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[†] National Research Council Fellow in Medical Sciences.

[1] Dalldorf, G., and Sickles, G. M., *Science*, 1948, **108**, 61.

TABLE I.

Isolation of Virus and Appearance of Neutralizing Antibodies in Patients from Southern New England During August October, 1948.

Patient*	Age	City	Diagnosis	Initial spinal fluid		Results of tests for virus		Neutralization of new virus by patient's convalescent serum
				WBC per cu mm	Ratio P : M	In monkeys (polio virus)	In newborn mice (new virus)	
1	9	New Haven	N.P.P.	31	33:67	—	+	+
2	5	” ”	A.M.	36	30:70	—	+	+
3	5	” ”	N.P.P.	27	7:93	—	+	+
4	11	Hartford	N.P.P.	125	54:46	—	+	+
5	32	”	N.P.P.	92	32:68	—	+	+
6	12	Providence	A.M.	600	15:85	—	—	+
7	6	New Haven	N.P.P.	129	25:75	—	—	+
8	18	Hartford	N.P.P.	140	74:26	—	—	+
9	6	New Haven	A.M.	42	47:53	—	—	+
10	12	Providence	N.P.P.	90	0:100	N.T.	N.T.	+
11	13	New Haven	A.M.	46	63:37	+	—	+
12	12	Stamford	N.P.P.	130	44:56	+	—	—
13	12	New Haven	P.P.	3	0:100	—	—	—
14	9	” ”	P.P.	43	70:30	—	—	—
15	13	Providence	P.P.	200	80:20	—	—	— (INC.)
16	14	”	A.M.	142	49:51	—	—	—
17	15	Hartford	N.P.P.	95		—	—	—
18	14	New Haven	F.U.O.	3	0:100	N.T.	+	+
19	26	” ”	F.U.O.	N.T.		N.T.	+	+

P : M—Ratio of Polymorphonuclear to Mononuclear Cells.
N.P.P.—Non-paralytic Poliomyelitis.
A.M.—Aseptic meningitis, cause unknown.
F.U.O.—Fever of Unknown Origin.
P.P.—Paralytic Poliomyelitis.
N.T.—Not Tested.
INC.—Incomplete, acute sample 6 days after onset was negative, convalescent sample not tested.

* We are indebted to the following physicians for their aid in obtaining specimens from these patients: Drs. Edward West and Alex A. Jaworski, Charles V. Chapin Hospital, Providence, R.I., Dr. F. W. Burke, James W. McCook Memorial Hospital, Hartford, Connecticut; and Dr. Richard Shay, Englewood Hospital, Bridgeport, Connecticut.

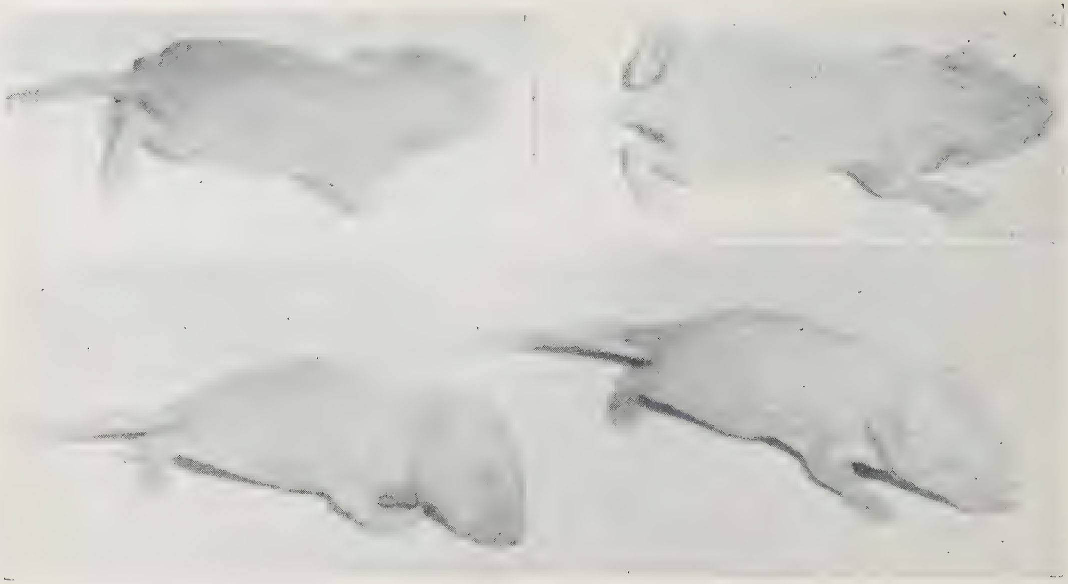

FIG. 1.

Two litter mates, the mouse on the left with paralysis of both legs and right arm (wrist-drop), the one on the right without signs of disease. Virus was inoculated intracerebrally and intraperitoneally on the 3rd day of life. Illness was first noted 6 days later when the photographs were taken.

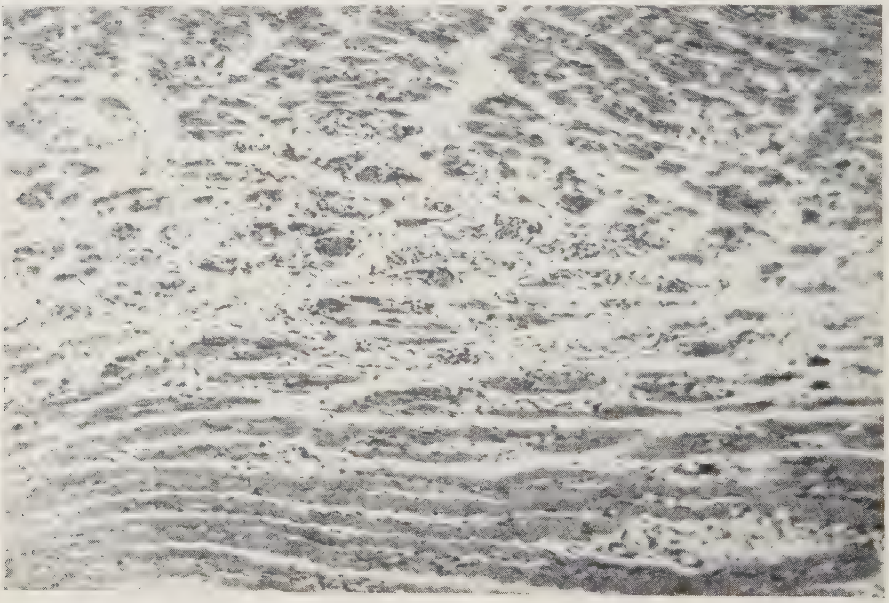

FIG. 2.

Myositis in paralyzed limb of 8-day-old mouse, sacrificed on the first day of paralysis. This animal was inoculated by the intracerebral and intraäbdominal routes within 24 hours after birth. The photomicrogrph was taken at 155 × magnification.

(iii) the convalescent sera of the 3 patients diagnosed as paralytic poliomyelitis and the serum of the 2 non-paralytic patients from whom poliomyelitis virus was isolated, all failed to neutralize the virus. The results of complement fixation tests with sera from these patients indicated that mumps virus was not the etiological agent of their illnesses.

(2) *Description of the experimental disease and some properties of the virus.* Swiss mice generally between the 1st and 2nd day of life have been used. Infectivity titers have ranged from 10^{-5} to 10^{-6} with suspensions of brain and slightly higher with suspensions of muscle. The mice have been inoculated intracerebrally and/or intraperitoneally. Signs of disease have appeared within 2 to 10 days, manifested by weakness and paralysis of one or more extremities (Fig. 1) and followed by death generally within 24 hours. In very young mice marked ataxia may be the only sign before death. The outstanding pathological finding has been an extensive myositis in the skeletal muscles especially of the limbs (Fig. 2). In some mice lesions have been noted in the heart muscle and in the brain.

Aerobic and anaerobic cultures of suspensions of tissues used for passage have shown no bacterial growth on ordinary media. The agent as it occurs in human feces and in mouse brain used for passage, has been passed through a tested, Corning, bacterial, glass-fritted filter. The agent is resistant to the action of ether, penicillin, streptomycin and chloromycetin. It has failed to produce disease in 3 week old mice, cotton rats, snowball rats, albino rats, hamsters as well as in young adult monkeys.

Studies on the distribution of the virus in newborn mice indicate that it is widespread at the time of paralysis; it has been recovered from the blood, brain, muscles, heart, liver, spleen and also from the intestinal contents. That the agent recovered from this latter source was not Theiler's TO virus was indicated by its lack of pathogenicity for 3 week old mice.

Preliminary data on sedimentation suggest that the agent is one of the smaller viruses. Thus, although some of the virus may be thrown down together with the particles which sediment at 18,000 r.p.m. for 30 minutes (6 inch rotor), most of the virus remains in the supernatant fluid. It may be sedimented readily at 36,000 r.p.m. for 60 minutes.

Neutralization tests have been carried out in newborn mice by the intraperitoneal route. It was found that the virus is readily neutralized by homologous antiserum prepared by repeated inoculation of mice or monkeys. It is not neutralized by antiserum from animals hyperimmunized against the following viruses; poliomyelitis (Lansing strain, North Carolina 1948 strain, Texas 1948 strain), Theiler's FA and GDVII strains of mouse encephalomyelitis virus, mumps, herpes, lymphocytic choriomeningitis, encephalomyocarditis, louping ill, Venezuelan equine encephalitis, and Newcastle disease.

(3) *Infection of chimpanzees.* As mentioned above, rhesus monkeys do not appear to be susceptible to infection with this virus when inoculated intracerebrally, intraperitoneally, intramuscularly, intracutaneously or subcutaneously. Two chimpanzees were each given one oral administration of the virus. No clinical signs of illness developed. However, virus was recovered from their feces for 12 days after the feeding and from their throats on the 5th, 6th and 8th days. Neutralizing antibodies, absent before exposure to virus, were present on the 14th and 28th days.

(4) *Accidental infection of man.* One of the physicians engaged in work with this agent developed a vague febrile illness of 8 days duration which was diagnosed as "fever of unknown origin." The only suggestion of involvement of the central nervous system was minimal stiffness of the back; a spinal tap was not done. Virus was recovered from the feces and nasopharyngeal washings during the acute illness. Neutralizing antibodies were not found in serum collected before or during the early acute phase of illness, but appeared in increasing titer during convalescence. The neutralization index on the 43rd day after onset of illness was 10,000.

(5) *Occurrence of the virus in "poliomyelitis" patients in Ohio, 1947 and North Carolina, 1948.* Samples of feces collected from different parts of the country and frozen since collection were also examined for the virus. It was not found in pooled specimens collected during 1944 from patients with paralytic poliomyelitis in New York City, nor from similar specimens collected from patients in

Los Angeles, California in 1948. These pooled samples were proven by monkey inoculation to contain poliomyelitis virus. The new virus, as well as poliomyelitis virus, was isolated from pooled fecal samples of 6 patients who had non-paralytic poliomyelitis in 1947 in Akron, Ohio. Both viruses were also present in pooled fecal samples collected in 1948 from patients with paralytic poliomyelitis in Winston-Salem, N. C. An ultracentrifuged concentrate of the Winston-Salem specimens had titers of 10^{-3} for poliomyelitis virus in monkeys and of $10^{-3.5}$ for the new virus in newborn mice.

(6) *Occurrence of the virus in extra-human sources.* The virus has been sought in samples of the sewage from 6 cities, 3 situated in Connecticut (Hartford, Norwalk, New Haven) and 3 in North Carolina (Greensboro, High Point, and Winston-Salem), and in non-biting flies trapped in 2 of these cities. In the summer of 1948 "mild poliomyelitis" appeared to be prevalent in Connecticut, and a severe epidemic of classical poliomyelitis occurred in North Carolina. As already mentioned, the new virus was isolated from patients in both areas.

The new virus was found in the sewage of all of the above 6 cities during some part of the summer and fall of 1948 and usually several serial samples from each city were positive. Serial sampling during the winter has for the most part been negative but the data are too few to indicate whether the occurrence of the virus in sewage follows the seasonal pattern of poliomyelitis virus.[2,3]

Tests of flies from the above areas for the presence of the new virus have not yet been completed, but thus far 2 isolations have been obtained, one from flies trapped in August, 1948 in Hartford, Connecticut and another from flies trapped in July, 1948 in High Point, N. C. In addition to studying this material from Connecticut and North Carolina, flies from Texas were also examined. These were trapped serially in 1948 during an epidemic of poliomyelitis in the lower Rio Grande Valley.[‡] From some batches of flies, separated according to species, both the new virus and poliomyelitis virus have been isolated. Other batches of flies have yielded either one virus or the other, and many batches have yielded neither virus. The species of flies which have given positive tests for both viruses are (a) *Musca domestica,* (b) *Phaenicia sericata* and *P. pallescens,* and (c) *Sarcophagula* and *Sarcophaga* spp. The two viruses (poliomyelitis and the new virus isolated in mice) even when obtained from the same batch of flies do not appear to be related by the tests we have thus far used (host range, virus neutralization by hyperimmune sera.)

(7) *Immunological types.* By means of cross neutralization tests, it has been found that two strains of the murine infecting virus isolated from patients in New Haven are related to each other as well as to a strain isolated from Hartford sewage. Furthermore a strain from Texas flies was found to be related to a North Carolina sewage strain, but not to the Connecticut strains. Thus each strain was readily neutralized by homologous hyperimmune sera, but antisera against Connecticut strains failed to neutralize the Texas virus, and, in similar fashion, Texas antisera had no effect on the Connecticut virus.

Summary. This paper reports the isolation of a filtrable virus from the feces of patients diagnosed either as non-paralytic poliomyelitis or aseptic meningitis and from 2 patients with "fever of unknown origin." The agent is similar to that reported by Dalldorf and Sickles[1] in producing paralysis with moysitis in newborn mice. The recovery of virus was correlated with the appearance of neutralizing antibodies in the patients' sera. At least two immunological types of the virus exist. The virus was widespread in this country during the summer of 1948 having also been isolated from the sewage of a number of

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[2] Trask, J. D., and Paul, J. R., *J. Exp. Med.*, 1942, **75**, 1.

[3] Melnick, J. L., *Am. J. Hygiene,* 1947, **45**, 240.

[‡] The flies in Texas were caught and identified by Dr. Richard P. Dow, Entomologist, and Staff, attached to the Dysentery Control Project, U. S. Public Health Service. They were collected as a part of field studies on epidemic poliomyelitis being carried out by this unit.

cities and from flies collected in widely separated areas. Subclinical infection may be produced in chimpanzees by oral administration of the virus. A laboratory worker has been accidently infected with the virus.

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17187. Effect of Phenergan (N-Dimethylamino-2-Propyl-1-Thiodiphenylamine, 3277 RP) on the Arthus Reaction in Rabbits.

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The so-called antihistaminic compounds have been of great value in that they have made possible the study of various biological phenomena, principally those related to histamine reactions, and the treatment of histamine-like allergies. As yet, none of the antihistaminics have been found to be effective in modifying the necrotizing type of allergic reactions. The lack of effect of pyribenzamine on the Arthus phenomenon and bacterial allergic reactions has been reported.[1] The present study is presented because an unusually potent antihistaminic, possessing, in addition, properties apparently unrelated to its antagonism to histamine, has been found effective in inhibiting the Arthus reaction.

The Fourneau compounds[3,4] and antergan[2] were modified to produce phenergan, N-dimethylamino - 2 propyl - 1-thiodiphenylamine, (3277 RP) which was extensively studied by Halpern.[5,6,7] In addition to being 30 times more effective than antergan in protecting guinea pigs against histamine, phenergan apparently protects capillaries against injury by various noxious stimuli.[8–12] The drug was kindly given for this study by Dr. B. N. Halpern. A quantitative technic for the induction of the Arthus reaction with known quantities of a single antigen and its homologous antibody has been described.[1,13] This method was employed in the present study.

Experimental. Arthus reactions were simultaneously induced in untreated control rabbits and in those treated with varying amounts of phenergan (25 to 100 mg/kg). In preliminary experiments, the Arthus reaction was induced in multiple sites in 9 albino rabbits by the local injection of antibody and of antigen as previously described.[1] It was found that 75 mg/kilo of phenergan in saline intramuscularly prevented the development of a severe Arthus reaction. The technic for producing the Arthus reaction was then modified by the administration of the antigen intraven-

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[1] Fischel, E. E., Proc. Soc. Exp. Biol. and Med., 1947, **66**, 537.

[2] Halpern, B. N., *Comp. r. Soc. de Biol.*, 1946, **140**, 361.

[3] Fourneau, E., and Bovet, D., *Arch. Intern. Pharmacol. et Ther.*, 1933, **46**, 178.

[4] Staub, A. M., *Ann. Inst. Pasteur*, 1939, **63**, 400, 485.

[5] Halpern, B. N., *Arch. Intern. Pharmacol. et Ther.*, 1947, **74**, 314.

[6] Halpern, B. N., *J. Allergy*, 1947, **18**, 263.

[7] Halpern, B. N., *Acta Allergologica*, 1948, **1**, 3.

[8] Halpern, B. N., and Cruchaud, S., *Comp. r. Soc. de Biol.*, 1947, **141**, 1038.

[9] Halpern, B. N., Hamburger, F., and Cruchaud, S., *Acta Allergologica*, 1948, **1**, 97.

[10] Halpern, B. N., and Cruchaud, S., *Comp. r. Acad. des Sciences*, 1947, **225**, 1194.

[11] Hamburger, J., Halpern, B. N., and Neel, Z., *Comp. r. Soc. de Biol.*, 1948, **142**, 183.

[12] Halpern, B. N., personal communication.

[13] Fischel, E. E., and Kabat, E. A., *J. Immunol.*, 1947, **55**, 337.

TABLE I.
Effect of Phenergan on Severity of the Reverse Arthus Reaction Induced Passively in Animals Injected Intravenously with 1.5 to 2.0 mg of Crystalline Egg Albumin Nitrogen, and 30 Minutes Later Injected Intradermally with Known Amounts of Anti-Egg Albumin.
Number of reactions of different severity related to total reactions in each group.

Severity of the reaction	Phenergan 100 mg/kg	Phenergan 75 mg/kg	Phenergan 50 mg/kg	Phenergan 25 mg/kg	Control animals
		0.22 mg AbN.			
0	0	0	0	0	0
±	0	2/26	0	0	0
+	1/20	20/26	3/20	0	4/38
++	18/20	4/26	13/20	4/4	5/38
+++	0	0	4/20	0	3/38
++++	1/20	0	0	0	26/38
		0.025 mg AbN.			
0	1/4	2/18	0	0	0
±	3/4	8/18	4/4	2/4	5/14
+	0	8/18	0	2/4	8/14
++	0	0	0	0	1/14

ously and the subsequent injection of antibody intracutaneously.[13] This procedure was found to result in a more rapidly developing and more hemorrhagic reaction than did the local administration of both antigen and antibody. The antigen was administered intravenously to 45 rabbits in amounts of 1.5 to 2.0 mg egg albumin nitrogen (EaN); 30 minutes later the antibody was injected intracutaneously in amounts of 0.22 and 0.025 mg antibody nitrogen (AbN). Untreated controls were injected simultaneously with phenergan treated animals. The treated group of animals received 2 doses of phenergan in amounts of 25, 50, 75 or 100 mg/kg intramuscularly. The first dose was given at the time the antigen was administered; and the second dose, 4 hours later. One of 6 animals given 100 mg/kg phenergan had convulsions after the second dose and died. The other animals showed some drowsiness, muscular incoordination and occasional muscular twitchings with the dosages of 75 or 100 mg/kg but recovered completely in a few hours. The skin reactions were observed at 6 and 24 hours. In this series of rabbits the most acutely severe reactions appeared at 6 hours after the injections of the antibody. The reactions were graded as follows:

0 = no reaction; + = petechial hemorrhages of an area less than 1 cm in diameter with minimal edema; ++ = less than 1 cm of confluent intracutaneous hemorrhage or 1 cm of petechial hemorrhage with edema over a somewhat larger area (2 cm); +++ = 1 cm of confluent hemorrhage or 2 cm of petechial hemorrhage with moderate edema; ++++ = 1.5 cm or more of confluent hemorrhage, with moderate to severe edema. The results are presented in Table I.

In Table I, it is apparent that phenergan has a definite inhibitory effect on the severity of the reaction at the higher concentrations of antibody (0.22 mg AbN). When only 0.025 mg AbN was employed, there did not appear to be any significant difference between the control animals and those receiving phenergan. It is much more difficult to compare minimally severe reactions employing the criteria used in this study. In consideration of the sedative and anesthetic side effects of phenergan, two animals were given respectively procaine and pentobarbital intramuscularly and were given both antigen and antibody locally. The severity of the reactions in these animals, although not tabulated, was similar to that of untreated control animals. In Table I, with the maximum amount of antibody (0.22 mg AbN), 29 out of 38 reactions, or 75%, in the control group were graded +++ or more. In the treated group, only 5 of 70 reactions, or 7%, were similarly graded. Since the dose of phenergan administered here is far greater than the dose of pyribenzamine used in the previous study (10 mg/kg),[1] it seemed relevant to determine whether pyribenzamine given in comparable amounts would affect the Arthus reaction.

Two rabbits were given 25 mg/kg and 75 mg/kg respectively of pyribenzamine intramuscularly in a single dose and died shortly thereafter with severe convulsions. To investigate further the activity of phenergan, the effect of the drug was studied on the skin reaction of the rabbit to the streptococcal erythrogenic toxin. Two control animals observed 24 hours after the injection of 2 skin sites with 550 skin test doses of toxin showed areas of edema and erythema 1.5 cm in diameter. Milder edema and erythema resulted after the injection of 55 skin test doses into two other sites. In contrast, 2 other animals treated with 75 mg/kg of phenergan every 4 hours for 3 doses showed no reaction to either the 55 or the 550 skin test doses of erythrogenic toxin. The observation that phenergan inhibits the effect of the erythrogenic toxin is consistent with the view that the drug appears to protect the capillary bed from injury.

Discussion. The severity of the Arthus reaction can be related to the amount of precipitin available for union with antigen locally.[13,14] The primary site of the injury due to the antigen-antibody reaction is probably the small blood vessels and capillaries.[15] Certain observations indicate that the action of phenergan is on these small blood vessels. Halpern and his associates have shown that the drug, in doses of 20 mg/kg, can protect rabbits against pulmonary edema induced by the injection of such unrelated agents as epinephrine and chloropicrin, a war gas.[8-10] Furthermore, Hamburger *et al.*[11] have studied the effect of phenergan on experimental orthostatic albuminuria and hematuria in rabbits, and found that the animals were completely protected by doses of 20 mg/kg. The inhibition of the Shwartzman reaction in rabbits has been noted recently.[12] These observations suggest that the effect of the drug on the Arthus reaction may well be a consequence of its ability to decrease capillary damage in the presence of nonspecific irritants. As such, the mechanism of action would be on the vascular bed and have little relation to the occurrence of an antigen-antibody reaction.

The dosages of phenergan employed in this study (25 to 100 mg/kg of body weight) were not lethal for the rabbit except for the highest dose, 100 mg/kg, which caused death in 1 of 6 rabbits. These doses are far greater than the amounts used therapeutically in humans, and, indeed, are the highest reported in animals. Halpern and Hamburger used 20 mg/kg effectively against experimental pulmonary edema and orthostatic albuminuria. The dose of phenergan which is effective as an antihistaminic is still smaller.

Summary. The effect of phenergan on the Arthus reaction was investigated, employing a quantitative method for the induction of the reaction. In large but non-lethal doses, the drug was found to inhibit appreciably the development of the more severe Arthus reactions. It is suggested that the action of the drug on the Arthus reaction is related to its effect on capillary permeability. A limited study of the inhibition by phenergan of the toxic effect of a large quantity of streptococcal erythrogenic toxin appears to support this view.

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[14] Opie, E. L., *J. Immunol.*, 1924, **9**, 231.

[15] Abell, R. G., and Schenk, H. P., *J. Immunol.*, 1938, **34**, 195.

17188. Effect of Isuprel Upon Tolerance of the Eviscerate Rat for Glucose.

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Epinephrine[1] and posterior pituitary extracts[2] have similar effects upon the tolerance of the eviscerate rat for glucose. When a solution of glucose without insulin is administered to the eviscerate rat, the addition of epinephrine or posterior pituitary extract to the solution has little or no effect upon the level of blood glucose during the first 2 hours but over a period of 24 hours there is an increase in glucose tolerance. When insulin is given with glucose, the addition of either epinephrine or posterior pituitary extract caused a rapid decrease in glucose tolerance.

It was proposed to extend these studies by testing a derivative of epinephrine having a depressor action upon blood pressure. In the present study, isuprel (isopropylepinephrine) was found to behave as epinephrine and the pressor principle of the posterior pituitary in causing a striking decrease in the glucose tolerance of the eviscerate rat given glucose and insulin.

Methods. The two-stage procedure of evisceration has been described.[3] When male rats of the Sprague-Dawley strain reached 250 ± 2 g, they were anesthetized (cyclopal) and subjected to the second stage of evisceration. Intravenous infusions of solutions containing glucose (C.P. Dextrose, Merck) and insulin (Regular Insulin, Lilly) were made by a continuous injection machine which delivered fluid into the saphenous vein of the right hind leg at the rate of 20 cc per 24 hours for each of 6 rats simultaneously. The glucose load was 64 mg per 100 g of rat per hour and the insulin dose was 4 units per 24 hours per rat. The temperature was constant at 26.5 ± 0.5°C. At the end of 2 hours, jugular vein blood was analyzed for glucose by the method of Miller and Van Slyke.[4]

Experiments and results. In Experiment 1, isuprel was administered to one rat of each of 12 pairs by adding it to the infusion fluid in a concentration of 1:25,000. In Exp. 2, isuprel was administered to one rat of each of 12 pairs by adding it to the infusion fluid in the concentration of 1:10,000. As shown in Table I, the addition of isuprel caused a striking decrease in glucose tolerance in both experiments.

Discussion. The effects of isuprel upon blood pressure were not studied under these experimental conditions. Although this compound has been described[5] as having a vasodepressor action in experimental animals, it would be unsafe to assume that its vasomotor effects are dissimilar from those of epinephrine and posterior pituitary extracts in the eviscerate rat. It was noted that isuprel caused the skin to become pink, suggesting peripheral vasodilatation, in contrast to the pallor caused by the peripheral vasoconstriction which occurs during the administration of epinephrine or of posterior pituitary extract under identical conditions. The mechanism whereby these drugs affect glucose tolerance in the eviscerate rat is not known but the possibility that it is secondary to circulatory changes must be considered.

Summary. Eviscerate rats (250 g) were given continuous intravenous infusions of glucose and insulin during a period of 2 hours. The glucose load was 64 mg of glucose per 100 g of rat per hour and insulin was given at the rate of 4 units per rat per 24 hours. The solution of glucose and insulin was infused at the rate of 20 cc per 24 hours. The addition

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[1] Ingle, D. J., and Nezamis, J. E., *Am. J. Physiol.*, 1949, **156**.

[2] Ingle, D. J., and Nezamis, J. E., *Am. J. Physiol.*, 1949, **156**.

[3] Ingle, D. J., *Exp. Med. and Surg.*, 1949, **7**, 34.

[4] Miller, B. F., and Van Slyke, D. D., *J. Biol. Chem.*, 1936, **114**, 583.

[5] Lands, A. M., Nash, V. L., Dertinger, B. L., Granger, H. R., and McCarthy, H. M., *J. Pharm. and Exp. Therap.*, 1948, **92**, 369.

TABLE I.
Effect of Isuprel upon Level of Blood Glucose at the End of 2 Hours in Eviscerate Rats Given Glucose and Insulin by Constant Injection.

Exp.	Conc. isuprel	No. rats	Avg blood glucose	Avg*	Diff. in avg	Diff.*	Diff. / diff.*
1	1:25,000	12	161	7.9			
	0	12	84	6.9	77	10.5	7.3
2	1:10,000	12	185	9.1			
	0	12	84	5.2	101	10.5	9.6

* Standard deviation.

of isuprel in concentrations of 1:10,000 and 1:25,000 caused a marked decrease in the glucose tolerance of the eviscerate rats.

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17189. Effect of Testosterone Propionate on Phosphatases in the Seminal Vesicle and Prostate of the Rat.*

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The discovery by Kutscher and Wolbergs[1] that the human prostate gland and ejaculum contain high concentrations of acid phosphatase provided the stimulus for a series of investigations of this enzyme in the tissues and secretions of the male reproductive tract.

Gutman and Gutman[2] showed that the high level of acid phosphatase (AcP-ase) as well as alkaline phosphatase (Alp-ase) in the prostatic tissue of the adult rhesus monkey is not present in the prepubertal animal, but that these enzymes can be raised to the adult level by the injection of androgen.

Pazos and Huggins[3] found that administration of androgen to starving, castrate immature dogs caused an 8 to 11-fold increase in AcP-ase activity of the prostate gland over that of a control animal. The Alp-ase in the glands of these dogs decreased 15-45%.

When Gutman and Gutman[4] studied the rat, however, they found that the levels of both AcP-ase and AlP-ase in the prostate gland were extremely low compared to levels in the primates studied. Administration of testosterone to normal adult male rats produced no change in the activity of either enzyme.

Atkinson[5] has shown histochemically that the AlP-ase in the stromal elements of the mouse seminal vesicle diminishes after castration and returns to normal after androgen treatment. Dempsey, Greep, and Deane[6] recently demonstrated the same phenomenon in the rat, also by histochemical means.

It was decided to conduct investigations to (1) determine whether an enzymatic response to testosterone could be obtained in the

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† National Cancer Institute Predoctorate Research Fellow, National Institute of Health.

[1] Kutscher, W., and Wolbergs, H., *Z. physiol. Chem.*, 1935, **236**, 237.

[2] Gutman, A. B., and Gutman, E. B., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 277.

[3] Pazos, R., and Huggins, C., *Endocrinology*, 1945, **36**, 416.

[4] Gutman, A. B., and Gutman, E. B., PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 529.

[5] Atkinson, W. B., *Anat. Rec.*, 1948, **100**, 731.

[6] Dempsey, E. W., Greep, R. O., and Deane, H. W., *Endocrinology*, 1949, **44**, 88.

TABLE I.
Changes in Seminal Vesicle Following Castration and Treatment with Testosterone Propionate.

Experimental stage	No. of rats*	Wt of one seminal vesicle (mg)	AcP-ase†	AlP-ase†	Dry wt (%)
Normal	4	160 (150-167)	17 (16-18)	34 (22-46)	20
Castrate 4 days	3	120 (112-130)	13 (11-15)	25 (20-29)	19
,, 8 ,,	3	80 (77-86)	12 (11-14)	17 (13-20)	22
Testosterone 4 days	3	375 (227-522)	14 (12-17)	33 (31-34)	20
,, 8 ,,	3	275 (273-279)	18 (14-22)	33 (28-37)	21

* Pertains to enzyme values only. Gland weights and dry weights taken from two rats in each group.

† Expressed as Huggins-Talalay units per 100 mg dry tissue. Figures in parentheses indicate ranges.

rat prostate similar to that obtained in the monkey and in the dog, (2) to provide quantitative support for the histochemical findings on the AlP-ase of the seminal vesicle, and (3) to provide information on the hitherto uninvestigated AcP-ase in the seminal vesicle. An experiment was designed, therefore, to study both AcP-ase and AlP-ase in homogenates of prostates and seminal vesicles of normal rats, castrate rats and in castrate rats receiving testosterone propionate.

Methods. The animals used were male rats of the Sprague-Dawley strain, 2½ to 3 months old. The rats were divided into 3 groups: (1) Normal animals, untreated; (2) Castrates, untreated; (3) Castrates injected subcutaneously with 600 γ testosterone propionate per day in 2 daily doses. Animals of groups (2) and (3) were killed 4 and 8 days following castration.

At autopsy the ventral lobe of the prostate was removed and weighed. The tissue was laid on moist, hard-surfaced filter paper and minced with a scalpel blade to rupture the majority of the gland lobules and permit escape of the contained secretion. The chopped tissue was washed in cold glass-distilled water, blotted on filter paper, weighed, and transferred to a chilled glass homogenizing tube. One seminal vesicle was removed and separated from the adherent coagulating gland; the secretion was expressed, and the gland was washed with cold saline solution, blotted, and transferred to an homogenizing tube.

Tissues were homogenized in glass distilled water, one per cent homogenates being used throughout the study. Solids and water content of the tissues were determined by drying pieces of tissue or homogenates at 75°C for 24 hours.

The Huggins-Talalay method[7] of phosphatase estimation, modified as previously reported,[8] was used on all tissues. This method measures phenolphthalein liberated from phenolphthalein phosphate. Both AcP-ase and AlP-ase were determined on tissues from at least three animals in all stages studied. The sequence in which the experiments were made was randomized in order to distribute any variation caused by minor differences in technic and condition of the animals due to seasonal factors.

Results. Seminal Vesicle. After castration the seminal vesicle rapidly loses weight, undergoing a 25% reduction in 4 days and reaching a level 50% below the pre-castration level by 8 days. The AlP-ase activity is reduced 30% in the first 4 days after castration, reaching a level 50% below the normal by the eighth day. The proportionality between weight loss of the gland and loss of enzyme activity is striking. Enzyme activity is expressed in terms of activity per unit weight of dry tissue, however, and the reduction in activity is real, not merely a reflection of the reduction in size of the gland.

The AcP-ase follows a trend slightly different from the gland weight and the AlP-ase, being reduced in activity by 25% after 4 days of castration, then undergoing very little further reduction through the eighth day.

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[7] Huggins, C., and Talalay, P., *J. Biol. Chem.*, 1945, **159**, 399.

[8] Stafford, R. O., McShan, W. H., and Meyer, R. K., *Endocrinology*, 1947, **41**, 45.

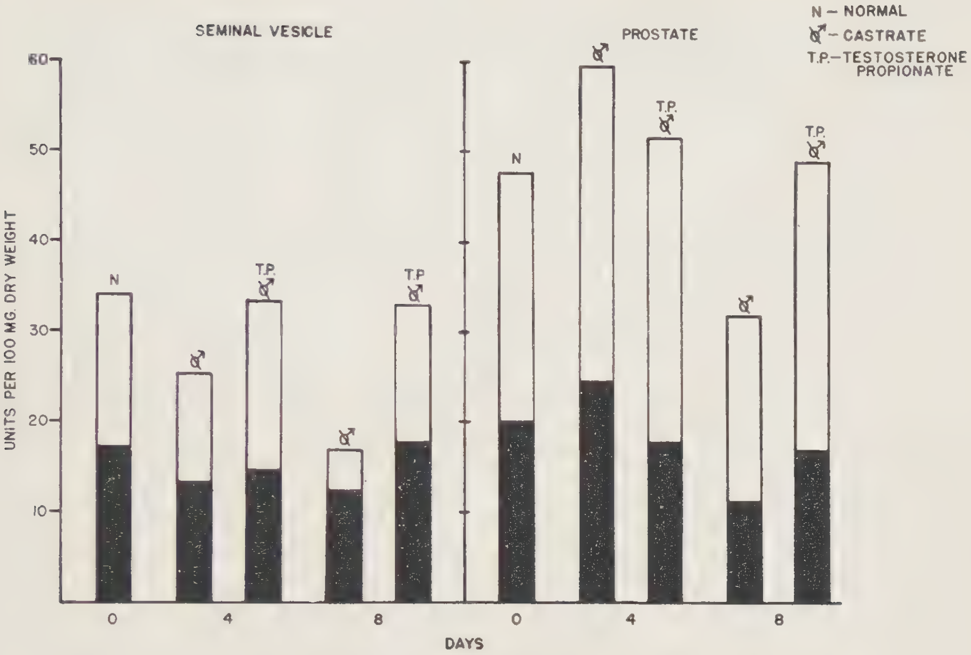

FIG. 1.

Changes in phosphatase activity produced by castration and treatment with testosterone propionate. AlP-ase activity is represented by total height of the bars. AcP-ase activity is represented by the black portions of the bars.

In the castrate animals receiving testosterone propionate, the androgen largely prevents the castration changes in weight and enzyme activity. The AlP-ase is maintained at almost exactly the same level in the androgen-treated animals as in the normals, while the AcP-ase activity is fully restored to the normal level by the eighth day of treatment. These data are presented in Table I and Fig. 1. The results clearly show that deprivation of androgen results in a loss of activity of both phosphatases in the seminal vesicle and that replacement therapy with testosterone propionate restores the activity.

Prostate Gland. The enzyme pattern in the prostate is similar to that obtained in the seminal vesicle with one major difference. While the castration effects are manifested in the seminal vesicle by the fourth day after castration, in the prostate the effects are not clearly shown until the eighth day. There is greater variability in the prostate values, probably caused by the variation in amount of secretion present in the tissues, as the technic for removal of the secretion may not be completely effective.

By the fourth day after castration, 2 out of 3 animals did not show significant losses of weight in the ventral lobe of the prostate, while the AcP-ase and AlP-ase actually increased in activity by 20 and 25% respectively. By the eighth day, however, the weight of the gland was about one-third of the normal weight and the AcP-ase and AlP-ase activities were reduced by one-half and one-third respectively.

When testosterone propionate was administered to the castrate animals, the AlP-ase of the gland was restored to normal and the AcP-ase activity was partially restored. The dose of testosterone propionate used was sufficient to cause great prostatic hypertrophy by the eighth day. These data are presented in Table II and in Fig. 1.

Histochemical treatment (Gomori[9]) of the prostate to demonstrate localization of the AlP-ase revealed a possible reason why

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[9] Gomori, G., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 23.

TABLE II.
Changes in Prostate Gland Following Castration and Treatment with Testosterone Propionate.

Experimental stage	No. of rats*	Wt of ventral prostate (mg)	AcP-ase†	AlP-ase†	Dry wt (%)
Normal	7	327 (303-366)	20 (14-28)	48 (30-64)	16
Castrate 4 days	6	266 (168-318)	24 (18-33)	59 (53-64)	15
,, 8 days	6	103 (75-140)	11 (7-13)	32 (25-48)	22
Testosterone 4 days	6	434 (376-487)	18 (17-21)	51 (38-76)	16
,, 8 ,,	6	728 (608-881)	17 (12-23)	50 (42-62)	17

* Pertains to enzyme values only. Gland weights and dry weights determined on 3 animals in each group.

† Expressed as Huggins-Talalay units per 100 mg dry tissue. Figures in parentheses indicate ranges.

the prostatic AlP-ase increases in concentration under the influence of androgen. The enzyme is localized in the epithelial elements of the gland, a positive phosphatase response being found at the basal and luminal borders of the epithelial cells. Since the androgen is trophic chiefly to the epithelium in this gland, causing an increased epithelial height, it is readily seen that androgenic stimulation should cause a relative increase in that amount of enzyme activity per unit weight of tissue.

Discussion. These data indicate that, contrary to previous belief,[4] the phosphatases in the prostate gland of the rat respond to androgen in a manner similar to that in the monkey and the dog. The quantitative data on the seminal vesicle presented here confirm the histochemical observations of Atkinson[4] and of Dempsey *et al.*[5] on the rat.

Davis, Meyer, and McShan[10] have shown that the succinic dehydrogenase (SDH-ase) and cytochrome oxidase in the prostate and seminal vesicle of the rat vary with the androgen supply in a manner somewhat similar to that shown here for the phosphatases. They found that in the castrate animal, the SDH-ase activity drops off as the weight of the glands decreases and returns when testosterone is administered. There is, however, one important difference, in the response of the two sets of enzymes studied. In both studies, dosage of testosterone used was sufficient to cause hypertrophy of the glands beyond the normal state. When this happened in the SDH-ase—cytochrome oxidase study, the enzyme activity rose beyond the level found in normal animals. In the case of the phosphatases, however, when the weight of the prostate was increased to more than twice that of the normal gland by 8 days of androgen treatment, the phosphatase activity did not rise beyond the normal level. The seminal vesicle under the same conditions hypertrophied to 70% above the normal weight, but the phosphatase activity did not rise above normal.

This phenomenon explains why Gutman and Gutman[4] were unable to show a response of the phosphatases to testosterone in their experiments; they were using adult, intact animals which presumably were at the maximal level of prostatic phosphatase activity before testosterone injections were begun.

The observations that the seminal vesicle is more sensitive to androgen deprivation than the prostate, morphologically[11] and with respect to oxidative enzyme activity[10] is borne out here again with respect to the phosphatases. While the seminal vesicle shows marked decrease in weight and phosphatase activity by the fourth day after castration, the prostate does not show a clear-cut response until sometime after this stage.

Summary. The acid and alkaline phosphatases of seminal vesicles and prostate glands of rats were estimated quantitatively in normal animals, castrates, and castrates receiving testosterone propionate.

In both glands the enzymes decrease in activity after castration, are restored to essentially normal levels by androgen therapy. The seminal vesicle is more sensitive than the prostate gland in this respect.

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[10] Davis, J. S., Meyer, R. K., and McShan, W. H., *Endocrinology*, 1949, **44**, 1.

[11] Korenchevsky, B., Dennison, M., and Brovsin, I., *Biochem. J.*, 1936, **30**, 558.

Androgen therapy caused hypertrophy of the glands beyond normal size, but under the conditions of these experiments, the acid and alkaline phosphatase activity was not increased beyond normal levels.

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17190. Investigations on the Use of Eserine for the Differentiation of Mammalian Esterases.*

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In most mammalian tissues there exist 3 types of esterase[1] capable of facilitating the hydrolysis of noncholine aliphatic esters, *i.e.*, ali-esterase,[2] pseudo-cholinesterase[1] (s-type)[3] and true cholinesterase[1] (e-type).[3] Most esters of aliphatic acids can be hydrolysed by enzymes from two of these 3 groups, certain non-choline esters (*e.g.*, triacetin) seem to be hydrolysed by enzymes of all 3 types[4-6] and, further, esterases of all 3 types are present in most crude tissue preparations. To distinguish between that portion of the hydrolysis of aliphatic esters which is due to the cholinesterases and that portion which is due to the ali-esterases in tissue preparations, Easson and Stedman[7] employed low concentrations of eserine or prostigmine to inhibit selectively any activity due to the cholinesterases (ChE's). Later investigators, *e.g.*, Richter and Croft,[2] Mendel and co-workers,[1] and others, also found low concentrations of eserine to be a very satisfactory tool for differentiating cholinesterase and ali-esterase activity. Thus it has been found that a concentration of 10^{-6}M eserine inhibits completely, or almost completely, the activities of both the true and the pseudo-ChE's of most mammalian tissues while the activity of the ali-esterases remains relatively unaffected by as much as 100 times this concentration.

Recently, however, McNaughton and Zeller[8] have concluded from their observations that "the use of eserine to decide whether a hydrolysis is catalysed by a cholinesterase or another esterase is of limited value because the s-type (pseudo-cholinesterase) is inhibited by this substance only in the presence of acetylcholine but not in that of ethyl chloroacetate." The evidence obtained in the present investigation has indicated, however, that eserine in low concentrations (10^{-6}M) can, in fact, be used to inhibit completely the activity of pseudo-ChE toward ethyl chloroacetate (EClA) and that differentiation of cholinesterase activity from ali-esterase activity is therefore still possible by this means. Moreover, even though this concentration of eserine may not be adequate to effect complete inhibition of the ChE's from certain non-mammalian organisms,[9] in no case tested has the sensitivity of any cholinesterase to eserine been found to be altered significantly when one

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* Supported by a grant from the National Research Council of Canada.

[1] Mendel, B., and Rudney, H., *Biochem. J.*, 1943, **37**, 59.

[2] Richter, D., and Croft, P. G., *Biochem. J.*, 1942, **36**, 746.

[3] Zeller, E. A., and Bissegger, A., *Helv. chim. acta*, 1943, **26**, 1619.

[4] Bodansky, O., *Ann. N. Y. Acad. Sci.*, 1946, **47**, 521.

[5] Hawkins, R., Ph.D. Thesis, Toronto, 1947.

[6] Adams, D. H., and Whittaker, V. P., *Biochem. J.*, 1948, **43**, xiv.

[7] Easson, E. H., and Stedman, E., *Biochem. J.*, 1937, **31**, 1723.

[8] McNaughton, R. A., and Zeller, E. A., Proc. Soc. Exp. Biol. and Med., 1949, **70**, 165.

[9] Hawkins, R. D., and Mendel, B., *J. Cell. Comp. Physiol.*, 1946, **27**, 69.

TABLE I.
Inhibition of the Activity of Human Plasma towards EClA Brought about by Various Inhibitors.

Inhibitor	Conc. of inhibitor (M)	Inhibition of Bch hydrolysis (%)	Inhibition of EClA hydrolysis (%)
Eserine	2×10^{-6}	100	43*
	2×10^{-5}	100	45
DFP	4×10^{-8}	100	45
	1×10^{-6}	100	44
	1×10^{-4}	100	42

* With 12 different samples of human plasma the eserine-sensitive portion of the EClA hydrolysis varied from 7-45% (average ca 15%) of the total activity.

substrate is substituted for another, whether this substrate be a choline or a non-choline ester.

The observation of McNaughton and Zeller that EClA is split with great rapidity in the presence of human serum or suspensions of guinea pig parotid gland has been confirmed and this rapid hydrolysis has similarly been found to be only slightly inhibited by 10^{-5}M eserine. However, while these preparations have been shown[10,11] to contain relatively large amounts of pseudo-ChE, no evidence was presented by these authors[8] to substantiate their assumption that the high activity towards EClA is not due, in part at least, to the presence of other esterases. To investigate the nature of the enzyme or enzymes responsible for the hydrolysis of EClA by crude tissue preparations, the following experiments were carried out:

(1) To exclude the possibility that the low percentage inhibition of total EClA hydrolysis by 10^{-5}M eserine (a concentration which inhibits completely pseudo-ChE activity towards acetylcholine, benzoylcholine, tributyrin, etc.) might in some way be associated with the fact that the inhibition produced by this compound is 'competitive' and reversible[12] the crude preparation was incubated with diisopropyl fluorophosphonate (DFP), an irreversible inhibitor with very high affinity for pseudo-ChE.[13] In the presence of 5 x 10^{-8}M DFP, pseudo-ChE was completely inactivated, as indicated by the total absence of activity towards benzoylcholine (Bch), a substrate specific for the pseudo-ChE's of most mammalian tissues.[10] Despite the complete and irreversible inactivation of its pseudo-ChE, however, human plasma tested in the presence of this concentration of the inhibitor still displays a high activity towards EClA. Moreover, the degree of inhibition of total EClA hydrolysis produced by this concentration of DFP was almost identical with that observed with eserine in concentrations which also completely abolish the activity of human plasma towards benzoylcholine. Since evidently the portion of the hydrolysis of EClA which is insensitive to eserine is also insensitive to DFP, the eserine-insensitive portion of the EClA hydrolysis is most likely due to a type of esterase distinct from the enzyme responsible for the eserine-sensitive portion of the activity. This supposition is further confirmed by the fact that considerable increases in the concentration of either inhibitor fail to augment the inhibition appreciably (Table I), in contrast to the results which would be expected if only one enzyme, the pseudo-ChE, were responsible for the total activity displayed towards EClA.

(2) To prevent any ali-esterases from participating in the activity displayed towards EClA, preferential inactivation of these enzymes was effected by heating undiluted oxalated human plasma at 52-53°C for 60-90 minutes. Plasma treated in this manner still displayed 65-75% of the activity of the untreated plasma towards Bch and all of its residual activity towards EClA was inhibited by eserine. These results demonstrate that the eserine-insensitive hydrolysis of EClA is

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[10] Mendel, B., Mundell, D. B., and Rudney, H., *Biochem. J.*, 1943, **37**, 473.

[11] Mendel, B., and Rudney, H., *Science*, 1943, **98**, 201.

[12] Straus, O. H., and Goldstein, A., *J. Gen. Physiol.*, 1943, **26**, 559.

[13] Hawkins, R. D., and Mendel, B., *Brit. J. Pharmacol.*, 1947, **2**, 173.

TABLE II.
Effect of Various Concentrations of Eserine and DFP on the Activities Displayed towards EClA and Bch by Heated* Human Plasma.

Inhibitor	Conc. of inhibitor (M)	Inhibition of Bch hydrolysis (%)	Inhibition of EClA hydrolysis (%)
Eserine	1×10^{-6}	97	99
	1×10^{-7}	87	87
	1×10^{-8}	51	52
	1×10^{-9}	11	8
DFP	8×10^{-9}	87	90
	2.5×10^{-9}	43	43

* Heated at 53°C for 90 minutes.

due to an enzyme or combination of enzymes distinct from the pseudo-ChE. That the eserine-sensitive residual EClA hydrolysis by the heated plasma preparation was due to pseudo-ChE could be concluded from the following evidence: (a) various concentrations of eserine and DFP inhibited the activities towards EClA and Bch to the same degree (Table II); (b) when hydrolysis was measured in the presence of a mixture of both substrates (EClA and Bch) no addition of the separate activities was observed—rather, only an activity similar to that exhibited towards Bch alone was displayed; (c) exposure of the treated plasma to a temperature of 56-57°C for 30 minutes depressed the activities towards Bch and towards EClA to the same degree (76% and 73% respectively).

(3) To demonstrate conclusively that the pseudo-ChE does not contribute to the eserine-insensitive portion of the hydrolysis of EClA by crude tissue preparations, the activity of highly purified pseudo-ChE preparations towards EClA was also investigated. Using a 9000-fold purified preparation of the pseudo-ChE obtained from horse serum,[14] the ratio

$$\frac{\text{activity towards EClA (0.04M)}}{\text{activity towards Bch (0.006M)}}$$

was found to be about 1.5, as compared with a ratio of 130 with a sample of untreated horse serum. Moreover, although a low concentration (10^{-5}M) of eserine inhibits only a minute fraction of the activity of untreated horse serum towards EClA, the activity towards EClA of the pseudo-ChE purified from the same source is completely abolished by the same concentration of eserine. It is evident, therefore, that the major portion of the hydrolysis of EClA by horse serum is due to the presence of eserine-insensitive ali-esterases which are removed from the preparation upon extensive purification of the pseudo-ChE contained therein.

From the evidence obtained it can be concluded that the pseudo-ChE does hydrolyse EClA but only at about 1½ times the rate at which it hydrolyses Bch and, further, its activity towards EClA is inhibited just as effectively by eserine as is its activity towards Bch. While various crude enzyme preparations may exhibit extremely high activity towards EClA—the ratio

$$\frac{\text{activity towards EClA}}{\text{activity towards Bch}}$$

varying from 4 in some samples of human plasma to 130 in horse serum and over 1000 in rat plasma—only that small portion of the total hydrolysis which is inhibited by low concentrations of eserine can be attributed to a cholinesterase. The large eserine-insensitive portion of this activity would, on the other hand, seem to be due to a distinctly different enzyme, an ali-esterase. This ali-esterase, it might be noted, is probably not identical with the ali-esterases responsible for the eserine-insensitive hydrolysis of tributyrin and/or triacetin by tissue preparations, as is evidenced by differences in sensitivity to certain inhibitors (*e.g.*, DFP) and by the fact that human plasma displays little or no eserine-insensitive activity towards these substrates although exhibiting a large eserine-insensitive activity towards ethyl chloroacetate[8] and certain substituted acetates of nitrophenol.[15]

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[14] Strelitz, F., *Biochem. J.*, 1944, **38**, 86.

[15] Huggins, C., and Smith, D. R., *J. Biol. Chem.*, 1947, **170**, 391.

Investigations with two 'irreversible' inhibitors—DFP as a preferential inhibitor of pseudo-ChE and tri-o-cresyl phosphate (TOCP) as a preferential inhibitor of ali-esterases[16]—showed that these compounds, unlike eserine, cannot be used as a general means of distinguishing pseudo-ChE and ali-esterase activities: certain ali-esterases, *e.g.,* the ali-esterase of rat plasma hydrolysing EClA, exhibit abnormally high sensitivity to DFP, while others, *e.g.,* the ali-esterase of rat brain hydrolysing tributyrin, proved to be quite insensitive to TOCP. In other words, the portion of the total activity inhibited by low concentrations of DFP may not always be a reliable index of pseudo-ChE activity and similarly the portion of the total activity inhibited by TOCP may not always be a reliable criterion of ali-esterase activity. However, in all cases tested as yet the ali-esterases have proved to be much less sensitive to eserine than the cholinesterases of the same tissue. In fact, so far as is known, a concentration of 2×10^{-6}M eserine can be used to inhibit completely or almost completely the cholinesterase activities in mammalian tissue preparations while leaving those of the ali-esterases unaffected.

Summary. Recent observations by McNaughton and Zeller on the high 'eserine-insensitive' activity displayed towards ethyl chloroacetate by various crude preparations of pseudo-ChE have been confirmed but the experimental results reported here fail to substantiate their conclusion that the activity of pseudo-ChE towards EClA is insensitive to eserine. The evidence presented here has, in fact, indicated that the activity of any of the cholinesterases towards ethyl chloroacetate is inhibited by low concentrations of eserine, the remaining eserine-insensitive activity being due to ali-esterases. The value of eserine for distinguishing that portion of the hydrolysis of aliphatic esters which is due to the cholinesterases from that due to ali-esterases in crude tissue preparations has been re-affirmed. It would seem that of the three inhibitors tested (eserine, diisopropyl fluorophosphonate, tri-o-cresyl phosphate), only eserine can be relied upon to give a clear cut distinction between cholinesterase and ali-esterase activities in all cases.

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[16] Mendel, B., and Mortimer, E. M., Report to National Research Council of Canada, October, 1944.

17191. Histamine and Other Imidazole Compounds as Bacterial Growth Stimulators.*

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In the course of a study it was observed that small amounts of histamine markedly stimulated the growth of certain bacteria. This report represents typical results of a comparative study of this effect with histamine and other derivatives of the imidazole nucleus.

Materials and methods. The culture medium was composed of NaCl (0.5%), $MgSO_4 .7H_2O$ (0.02%), $(NH_4)H_2PO_4$ 0.1%, K_2HPO_4 (2.4%), KH_2PO_4 (0.6%), glucose (0.2%) and distilled water. The reaction of the medium was pH 7.0. It was passed through a sintered glass filter to sterilize. Solutions of the test substances in the salt-glucose medium were also sterilized by filtration. No significant drop in pH occurred during growth with this high phosphate buffer concentration. A stock strain of *Klebsiella pneumoniae* was

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† Abbott Fellow in the Department of Bacteriology, 1948-1949.

TABLE I.
Effects of Imidazole Derivatives on the Growth of *K. pneumoniae.*

Substance tested	Concentration $\times 10^{-5}$ M	Turbidity readings, time in hr					
		14	16	18	20	22	24
Histamine · 2HCl	50	9	34	96	130	130	130
	10	7	22	70	130	130	130
1 (+) Histidine · HCl	50	50	104	130	130	130	130
	10	19	62	116	130	130	130
5-(4)-amino-4(5)-imidazolecarboxamide · HCl*	50	0	14	40	110	122	122
	10	0	4	15	60	116	126
Imidazole-4,5-dicarboxylic acid*	50	21	74	120	123	123	123
	10	6	38	100	130	130	130
Imidazole*	50	0	2	10	37	98	130
	10	0	1	6	25	86	128
p-Aminobenzoic acid 20 γ/ml†		0	0	3	12	53	109
Control		0	0	4	19	66	112

* 5(4)-Amino-4(5)-imidazolecarboxamide was obtained from Dr. John M. Buchanan. The other compounds were from Eastman Kodak Co.

† With 0.1 and 1.0 γ/ml growth was of similar magnitude.

used in this study. The inocula, derived from 24-hour salt-glucose cultures, were washed once with and resuspended in phosphate buffer and diluted so that after inoculation each system contained approximately 50 viable cells per ml. All cultures were incubated at 37°C. The turbidity readings (filter No. 54, Klett-Summerson photoelectric colorimeter) represent averages of triplicate determinations.

Micro-determination of Histamine and Histidine. This method is an adaptation of the method of Macpherson[1] and was carried out in Klett tubes. The final volume of the reaction system was 6 ml and consisted of from 2 to 20 γ of histamine or histidine per ml, 3 ml of water, 0.4 ml of sulfanilic acid in 10% hydrochloric acid solution, 0.4 ml of 5% sodium nitrite, and 1.2 ml of 30% sodium carbonate added after 30 minutes standing at room temperature. Colorimetric readings were made immediately with the use of the No. 54 filter. This permitted the determination of 2 γ of either compound.

5(4)-Amino-4(5)-imidazolecarboxamide.[2] This substance was determined according to the method of Bratton and Marshall.[3] This method carried out in Klett tubes permits the determination of 2 γ of the compound.

Results. The results given in Table I clearly indicate the stimulatory effects of histidine, histamine, imidazole-4, 5-dicarboxylic acid, and 5(4)-amino-4(5)-imidazolecarboxamide. Histidine concentration of 2 x 10^{-5}M, and histamine, 5 x 10^{-5}M, also exercised definite stimulatory effects. Imidazole has but slight effect and p-aminobenzoic acid has no effect. Maximal growth and the logarithmic growth rates are approximately equal for all systems. At the times of maximal growths in the accelerated systems (18 to 20 hours), the turbidities were 5 to 10 times greater than that of the controls. The observed stimulatory effect is that of shortening the lag phase, and is not as apparent when large inocula are used.

During growth there was no measurable utilization of histamine or of 5(4)-amino-4(5)-imidazolecarboxamide. Histidine was utilized in proportion to growth. Histidine can serve as a sole source of carbon for growth in the absence of glucose while histamine cannot. For lack of a quantitative method, it was not determined whether or not imidazole-4,5-dicarboxylic acid and imidazole were utilized. In the case of histamine and 5(4)-amino-4(5)-imidazolecarboxamide, if utilized, the stimulatory effect must be due to amounts less than could be detected by the micromethods described above. Whether or not these effects have bearing on purine metabolism, or possess a vitamine-like function are under investigation.

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[1] Macpherson, H. T., *Biochem. J.*, 1942, **36**, 59.

[2] Shive, W., Ackermann, W. W., Bordon, M., Getzendaner, M. E., and Eakin, M. E., *J. Am. Chem. Soc.*, 1947, **69**, 725.

[3] Bratton, A. C., and Marshall, E. K., *J. Biol. Chem.*, 1939, **128**, 537.

Summary. Histamine and certain derivatives of imidazole nucleus have been found in trace amounts to exercise stimulatory effects on the growth of *K. pneumoniae*.

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17192. Mechanism of Inhibition of Glycogen Synthesis by Endotoxins of *Salmonella aertrycke* and Type I Meningococcus.*

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Endotoxins of *Salmonella aertrycke* and meningococcus were found to have a marked effect on carbohydrate metabolism as measured *in vivo*[1,2] and *in vitro*.[2,3] These observations indicated that the endotoxins act on both anaerobic and aerobic phases of carbohydrate metabolism. The present paper describes experiments, dealing with the effect of endotoxins *in vivo* and *in vitro* on the oxidation of pyruvate as related to glycogen synthesis.

Experimental. The inhibitory effect of crude Salmonella endotoxin on the synthesis of liver glycogen was studied in detail. The experimental conditions were similar to those previously described,[2] but the time of *in vivo* glycogen synthesis was extended to a period of 5 hours. Twelve rats were divided into 4 groups: the first group received no glucose and no toxin; the second group was given glucose by stomach tube but no toxin; while the third group received Salmonella endotoxin but no glucose; and the fourth received both glucose and endotoxin. The amount of Salmonella endotoxin was 9 mg per 100 g body weight, a dose which was lethal for starving rats in 18 to 36 hours. Glucose was given 1 hour after the intraperitoneal injection of the endotoxin, and all animals were sacrificed 5 hours after glucose administration. The method of removal of liver samples and detailed analytical procedures for metabolic intermediates have been reported elsewhere.[4] Analytical results in each group of rats agreed from animal to animal within ± 15% for triosephosphates, phosphopyruvate, hexose mono- and diphosphates and pyrophosphates, and within ± 10% for the rest of the intermediates. Results are summarized in Table I, where the analytical data are expressed in terms of micrograms of intermediates per gram of liver wet weight.

These analytical results confirmed previous findings[2] since even small doses of the endotoxin completely inhibited glycogen deposition. The lactic acid content of the livers of rats, injected with Salmonella endotoxin, was markedly increased, an effect which was similar to that observed in rabbits after the injection of endotoxins.[1] This finding itself indicated tissue anoxia, which, on the other hand, resulted in an inhibition of the function of the adenylic system. The inhibition of carbohydrate synthesis at the level of phosphopyruvate was suggested by the absence of

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* This investigation was supported by the U. S. Navy, Office of Naval Research, and the University of Chicago.

† Present address: Tulane U. School of Medicine, New Orleans, La.

[1] Kun, E., and Miller, C. P., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 221.

[2] Kun, E., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 496.

[3] Kun, E., *J. Biol. Chem.*, 1948, **761**, 174.

[4] Kun, E., and Abood, L. G., *Arch. Internat. Pharmocodyn. et Ther.*, (Gand, Belgium), 1949, **80**, 51.

TABLE I.
Effect of Salmonella Endotoxin on Intermediate Metabolites of the Rat Liver During Glycogen Synthesis. (Dose of endotoxin = 9 mg/100 g rat).

Metabolite	Normal starving	Normal fed glucose	Starving, inj. with endotoxin	Fed glucose and inj. with endotoxin
Glycogen	440	41,500	0	0
Glucose-1-PO_4	86	68	15	10
Glucose-6-PO_4	1,980	1,350	220	240
Fructose-6-PO_4	200	80	10	10
Fructose-1-6-diphosphate	187	180	220	230
Pentose-PO_4	130	128	74	30
Triose-PO_4-phosphorus[*]	17	31	50	75
Phosphopyruvic acid	68	15	0	0
Pyruvic acid	9.5	9.5	18	12
Lactic acid	37	148	310	390
Pyrophosphate phosphorus[†]	46	10	6.0	29
Phosphocreatine	90	1,470	500	800
Insoluble nucleic acid phosphorus[‡]	1,100	830	760	550

[*] Values for triose phosphate phosphorus express the alkali labile phosphorus of the Ba soluble, alcohol-precipitable fraction.

[†] Values for pyrophosphate phosphorus represent the 7 minute phosphorus, obtained by hydrolysis of the Ba insoluble fraction in 1 N HCl.

[‡] The nucleic acid phosphorus was determined by the trichloroacetic acid procedure of Schneider in the insoluble protein residue.[4]

this intermediate. On this basis the inhibition of glycogen synthesis could at least partially be explained by an inhibition of biological oxidation. This was already suggested by previous observations, which showed the decreased succinoxidase capacity of the tissues of rabbits injected with endotoxins.[1] The significance of the decrease of the insoluble nucleic acid phosphorus cannot be evaluated at present.

Since it is generally accepted that the main pathway of biological oxidation is by way of pyruvate oxidation,[5] it appeared to be of special interest to demonstrate pyruvic oxidase inhibition *in vitro*, which inhibition was suggested by the results obtained in animal tissues.

An alcohol precipitable fraction of meningococcal and Salmonella endotoxin proved to be a powerful inhibitor of pyruvate oxidation *in vitro*. The preparation of the inhibitory substance was carried out following a procedure used by Boivin and Mesrobeanu for extracting an antigenic carbohydrate-lipid complex from Gram negative bacteria (bibliography cited in [6]). The crude endotoxins were centrifuged at 4000 R.P.M. for 60 minutes at 0°. The supernatant mixture of soluble proteins was precipitated by the addition of equal volumes of ice cold 10% trichloracetic acid. The precipitate was sedimented by centrifugation and the supernatant neutralized to pH 7.0. This neutralized supernatant was dialyzed against running tap water for 18 hours. The final step was the addition of 8 to 10 volumes of cold 95% alcohol. A light flocculent precipitate settled down when the mixture was kept for 24 to 48 hours at 0°C. This alcohol precipitable fraction was centrifuged and after several washings with 95% alcohol was dried under a stream of warm air. The yield of this fraction was estimated to be 25 to 30 mg per liter of crude meningococcal endotoxin containing 1% solid material, and the yield from Salmonella endotoxin was 6 to 8 times greater. These materials could be resuspended in isotonic salt solution, yielding a slightly opalescent suspension.

Pyruvic oxidase was prepared by a method similar to that employed by Lehninger and Green.[7,8] Three grams of rat or rabbit liver were homogenized in 15 ml ice cold potassium

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[5] Vennesland, B., *Ann. Rev. Biochem.*, 1948, Vol. XVII, 227.

[6] Boor, A. K., and Miller, C. P., *J. Infect. Dis.*, 1944, **47**, 75.

[7] Lehninger, A. L., *J. Biol. Chem.*, 1945, **437**, 161.

[8] Green, D. E., Loomis, W. F., and Auerbach, V. H., *J. Biol. Chem.*, 1948, **389**, 172.

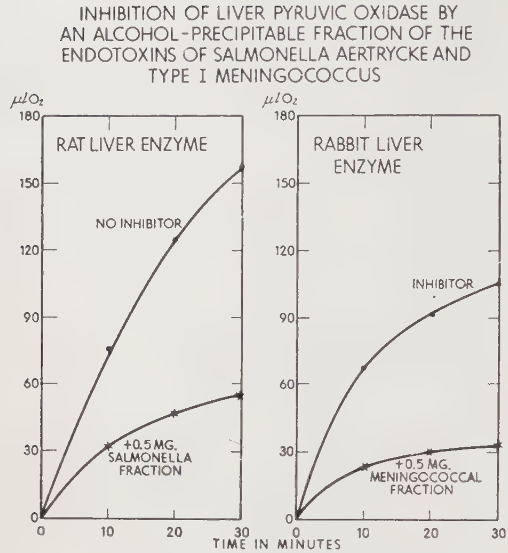

Fig. 1.

Each Warburg flask contained 300 mg enzyme, 0.5 ml 0.01 M adenosinetriphosphate, 10 mg sodium pyruvate as substrate, and 0.2 ml 20% sodium hydroxide in the center. The endotoxin fraction (0.5 mg) was suspended in 1 ml potassium medium. The final volume in each flask was made up to 3 ml. Oxygen consumption was measured at 37°C. The gas phase was air.

medium (containing 100 parts of 0.9% potassium chloride, + 21 parts 1.3% potassium bicarbonate, + 1 part 3.82% magnesium sulfate heptahydrate; to 120 ml of this mixture 1 ml of 2.1% potassium acid phosphate was added immediately before use). The homogenate was centrifuged and washed 3 times in the cold as described by Green[8] and finally resuspended to a volume, which contained 300 mg of original liver homogenate per ml. After the addition of adenosinetriphosphate and sodium pyruvate, oxygen consumption was measured with and without the addition of 0.5 mg of the endotoxin alcohol-precipitable fraction. In the presence of this fraction the oxygen consumption, due to the addition of pyruvate, was invariably depressed.

It was of interest that the alcohol precipitable fraction of meningococcal endotoxin was very toxic to rabbits (0.5 to 1.0 mg injected intravenously produced death in 24 hours) while rats and mice were susceptible to the Salmonella fraction (70 to 150 μg injected intravenously were lethal to mice and rats in 16 to 24 hours).

A similar difference was observed *in vitro*: the pyruvic oxidase of the rabbit liver was inhibited by the meningococcal endotoxin fraction, while the rat liver enzyme was sensitive to the Salmonella endotoxin preparation.

Summary. In vivo and *in vitro* experiments indicated that the inhibition of pyruvate oxidation occurred in animals injected with bacterial endotoxins. The inhibitory factor was probably identical with the toxic "antigéne complet" of Boivin and Mesrobeanu. It is suggested that the inhibition of pyruvate oxidation plays an important role in the toxic effect of endotoxins. The exact mechanism of the inhibition of pyruvate oxidation and the identification of the inhibitor as well as the biochemical nature of other components of the endotoxins remains to be further investigated.

The authors wish to thank Dr. C. Phillip Miller for the crude endotoxins prepared in his laboratory and for valuable criticism of this paper.

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17193. Effects of Certain Azo Dyes upon the Cockroach *Blattella germanica*.*

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Very little is known about the effects of carcinogens upon insects. In the present study the cockroach *Blattella germanica* (L.) was fed crude or synthetic diets containing varying amounts of p-dimethylaminoazobenzene (DAB), m′-methyl-*p*-dimethylaminoazobenzene (m′DAB) or aminoazobenzene (AAB). The effects of the dyes upon growth rate, maturity, survival, ability to reproduce and the occurrence of internal lesions were determined. Newly-hatched nymphs from single egg sacs were divided into groups of 5-7 and kept in wire-screened test-tube cages[1] at a temperature of 27-32°C. Food and water were given *ad libitum* and were replaced weekly. The nymphs were weighed under CO_2 anaesthesia at 10, 20, and 30 days of age. The age at maturation, the ability to reproduce, and the fraction surviving were recorded. All surviving adults were eventually dissected and examined for abnormalities.

Experimental. In 3 preliminary experiments DAB was fed at 0.03, 0.06, 0.1 and 0.2%, and m′DAB was fed at 0.06 and 0.2% of a crude diet consisting of dog biscuits (Friskies) 94%, corn oil 4% and cholesterol 1 or 0.1%. Both the growth rate and reproductive performance of these insects remained essentially normal. The insects were dissected at ages varying from 54 to 151 days. At these times abnormalities of the digestive tract were seen in 37 of the 43 adults exposed to the dyes, while none of 50 insects fed only the crude diet were affected similarly. A common condition was the occurrence of small black flecks in the walls of the esophagus, crop, mid-gut and hind-gut and in the tissue of the fat body. A characteristic finding in insects fed 0.1% DAB was a degeneration of the Malpighian tubules, which were sticky and tended to form clumps or nodules. In some cases small vesicles were present at the proximal ends of the tubules. In Experiment 1, 3 of 5 roaches fed 0.1% DAB were affected, in Experiment 2, 4 of 5 and in Experiment 3, 0 of 5. The entire digestive tracts of these affected insects were removed, imbedded by the dioxan-paraffin method, cut serially into sections 6-10 microns thick and stained with hematoxylin, with or without eosin counterstain. Photomicrographs were taken of representative lesions (Fig. 1-3).

In a more detailed study, the potent carcinogens DAB and m′DAB were fed for long periods of time at a level of 0.2% in both crude and synthetic diets, and the non-carcinogen AAB was fed at 0.2% in a synthetic diet (Table II). One hundred and four newly-hatched nymphs from 4 egg sacs were distributed among 18 cages and fed the 6 diets shown in Table I. Growth rate, reproduction, and survival were measured, and the adults were dissected at ages varying from 112 to 173 days. Some of the F_1 nymphs from each group were kept on the same diets as their parents, and were killed for examination at 97-101 days of age. In addition to the experimental insects, a total of 127 adults previously fed a variety of crude and synthetic diets not containing azo dyes were dissected at ages varying from 53 to 143 days.

The growth rate, reproduction, and survival of the roaches were not greatly affected by the three azo dyes, although growth was retarded

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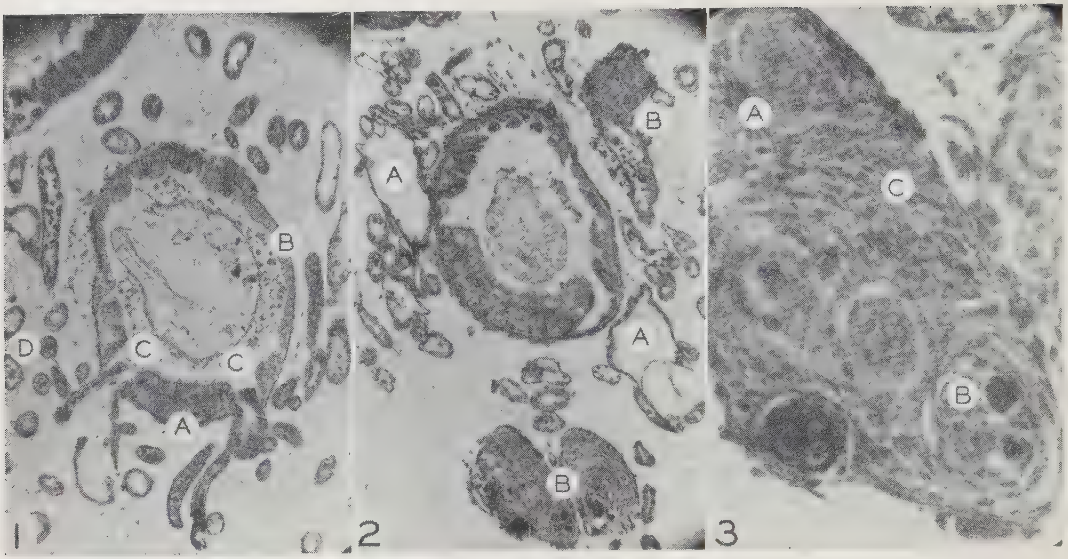
[1] Noland, J. L., Lilly, J. H., and Baumann, C. A., *Ann. Ent. Soc. Am.*, 1949, **42**, 63.



Cross-section of cockroach gut at the junction of mid-gut and hind-gut, showing lesions in the Malpighian tubules due to the feeding of azo dyes. A full description of this region as found in the normal cockroach is given by Miall and Denny.[2]

Fig. 1. Low Power. Cockroach previously fed 0.1% DAB for a short period of time. A—mid-gut. B—hind-gut. C—Origin of Malpighian tubules. D—Malpighian tubules in cross-section showing the beginning of degeneration.

Fig. 2. Low Power. Cockroach previously fed 0.1% DAB for a long time, showing the two characteristic types of lesions of the Malpighian tubules due to the azo dyes. A—Small vesicles formed by swelling of the tubules. B—Nodules formed by the fusion of adjacent tubules.

Fig. 3. High Power. Detail of a nodule shown in Fig. 2. A—Outlines of Malpighian tubules within nodule. B—Yellow granule of azo dye or its derivatives. C—Surrounding layers of hemocytes.

slightly when m′DAB was fed in the crude diet (age at maturity increased from 55 to 59 days) or DAB was fed in the synthetic diet (age at maturity increased from 49 to 64 days) (Table I). No consistent effect upon either reproduction or survival could be detected. The characteristic abnormality in insects fed the azo dyes in these experiments was the occurrence of large vesicles at one or more of the 6 points at which the Malpighian tubules arise from the intestine (see Fig. 1 and 2 for the location of these lesions). The vesicles ranged in size from one-fourth to 10 times the diameter of the mid-gut, and as many as 4 were observed in a single insect (Table II). The vesicles were usually thin-walled and filled with a light yellow fluid, but some were thick-walled and contained either a fluid or dark brown granules. In some individuals the proximal portions of adjacent Malpighian tubules were fused into nodules (Fig. 2 and 3). The extent of vesicle formation did not appear to be affected by the type of basal diet fed. Of the 3 dyes, DAB appeared to cause the most lesions, followed by m′DAB which was fairly active, and AAB which was slightly active (Table II).

Discussion. It is evident from these experiments that the cockroach has a much higher tolerance for the carcinogenic azo dyes than has the rat or mouse. 0.06% DAB or m′DAB in the diet seriously retards growth and prevents reproduction in the rat[5,6] while more than three times this level (0.2%) of either

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[2] Miall, L. C., and Denny, A., The Cockroach, London, 1886, 123.

[3] Noland, J. L., Lilly, J. H., and Baumann, C. A., *Ann. Ent. Soc. Am.*, in press, II.

[4] Noland, J. L., and Baumann, C. A., Proc. Soc. Exp. Biol. and Med., 1949, **70**, 198.

[5] Miller, J. A., Miner, D. L., Rusch, H. P., and Baumann, C. A., *Cancer Res.*, 1941, **1**, 699.

[6] Giese, J. E., Miller, J. A., and Baumann, C. A., *Cancer Res.*, 1946, **6**, 337.

TABLE I.
Growth Rate, Reproduction and Survival of Cockroaches Fed Various Azo Dyes.

Basal*	Diet supplement†	Wt at 30 days, mg	Age at maturity, days	Egg-sacs produced	Avg No. per egg-sac	Survival (fract.)
Crude	None	29	55	3	32	14/17
”	DAB	25	55	9	25	14/18
Synt.	”	31	64	1	29	11/18
Crude	m'DAB	20	59	5	28	9/12
Synt.	”	25	57	7	20	15/17
”	AAB	36	49	9	29	14/17

* The crude basal diet consists of "Friskies" dog biscuits 95% and corn oil 5%. The synthetic basal diet is "synthetic diet V."[3,4]

† All supplements were fed at 0.2% of the diet. DAB = *p*-dimethylaminoazobenzene; m'DAB = m'-methyl-*p*-dimethylaminoazobenzene; AAB = aminobenzene.

TABLE II.
Occurrence of Vesicles in the Malpighian Tubules of Cockroaches Fed Various Azo Dyes.

Diet*		Roaches dissected			Incidence of vesicles		
Basal	Supplement	Age, days	Sex M	Sex F	% incidence	Avg No. per roach	Avg diam.†
		First generation roaches.					
Crude	None	112-123	10	6	0	0	0
”	DAB	112-123	7	6	58	2.0	2.4
Synt.	”	112-123	6	3	89	2.1	2.5
Crude	m'DAB	112-123	6	4	30	1.3	1.2
Synt.	”	112-123	7	6	75	1.3	0.6
”	AAB	112-173	4	3	29	1.3	0.6
		Second generation roaches.					
Crude	None	97	0	10	0	0	0
”	DAB	97	5	10	100	1.7	2.5
Synt.	”	97	4	11	87	1.7	1.2
Crude	m'DAB	97	7	8	53	1.8	0.7
Synt.	”	97	3	10	46	1.7	0.5
”	AAB	101	7	6	69	1.6	0.6
		Unselected roaches.					
Crude	None	90	25	25	0	0	0
Fed 19 different synthetic diets		53-143	38	39	0	0	0

* For composition of diets, see footnote to Table I.

† Expressed as the ratio of the diameter of the vesicle to that of the mid-intestine, which is normally 0.75 mm in diameter.

compound had little or no effect upon the growth rate of the roach. Another difference is the relative order of activity of the 3 compounds fed. For the production of liver tumors in rats, m'DAB is much more active than DAB, and AAB is quite inactive[6,7] while for the production of Malpighian tubule lesions in the roach, DAB appeared to be more active than m'DAB, and AAB was also somewhat active. It is of interest that in the rat, tumors due to azo dyes are seldom found outside the liver[8] while in the roach, nodules or vesicles appeared only in the proximal region of the Malpighian tubules. Whether these latter lesions are tumors is doubtful; certainly no proof of autonomous growth is at hand.

An indication of the probable mechanism by which the lesions are formed was obtained by consecutive examination of serial sections

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[7] Miller, J. A., and Baumann, C. A., *Cancer Res.*, 1945, **5**, 227.

[8] Rusch, H. P., Baumann, C. A., Miller, J. A., and Kline, B. E., *A.A.A.S. Res. Conf. on Cancer*, 1945, 267.

of affected tissues. Apparently the azo dyes are absorbed from the gut into the hemolymph and are then taken up by the Malpighian tubules. If the tubules cannot excrete the dyes or their degradation products into the hind-gut, as is ordinarily done with other excretory substances,[9] the dyes or their derivatives will accumulate in the proximal portion of the tubules. Occlusion of the open ends of the tubules could lead to their swelling into vesicles (Fig. 2) as the absorption of fluid by the tubules continues. If the ends remain open, but the dyes stay within the tubules, local effects of the dye could cause the tubules to degenerate, become sticky and adhere to adjacent tubules.. Hemocytes then accumulate around the affected areas to form nodules (Fig. 2 and 3). Wigglesworth[10] cites similar cases in which the hemocytes of insects encapsulated internal parasites, or walled off areas of infection or irritation. An alternative explanation of nodule formation would be that the nodules are tumors which were formed by the action of the azo dyes upon the Malpighian tubules. However, there appears to be no evidence at present which would lend support to this view.

Summary. The azo dyes *p*-dimethylaminoazobenzene (DAB), m'-methyl-*p*-dimethylaminoazobenzene (m'DAB) and aminoazobenzene (AAB) were fed to the roach *Blattella germanica* (L) at varying levels in both crude and synthetic diets. A dietary level of 0.2% of the dyes had little or no effect upon growth rate, survival or reproduction of the insects. However, characteristic lesions at the proximal ends of the Malpighian tubules occurred in roaches fed the dyes. DAB was the most active, m'DAB was next, and AAB was least active of the compounds fed.

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[9] Wigglesworth, V. B., Insect Physiology, London, 1942, 310.

[10] Wigglesworth, V. B., Insect Physiology, London, 1942, 236.

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17194. Relation of Pantothenic Acid to White Blood Cell Response of Rats Following Stress.*

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Recent work has emphasized the role of the adrenal cortex in controlling the number of lymphocytes in the circulating blood following various forms of stress. Corticosterone produced a lymphopenia when injected into either adrenalectomized or intact mice.[1] The injection of the adrenocorticotropic hormone (ACTH) of the pituitary also produced a lymphopenia, provided the adrenals were present.[1,2] A deficiency of pantothenic acid causes lipid depletion and necrosis of the adrenal cortex.[3-6] Because of the importance of pantothenic acid to the physiological integrity of the adrenal cortex and in view of the influence of the adrenal cortex on the number of white blood cells, experiments have been done to determine the effect of diets adequate and deficient in pantothenic acid on the response of the white cells of rats subjected to stress.

Experimental. Young male rats of the Long-Evans strain, bred in the laboratory,

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* This research was aided by a grant from the National Vitamin Foundation.

[1] Dougherty, T. F., and White, A., *Endocrinology*, 1944, **35**, 1.

[2] Reinhardt, W. O., Aron, H., and Li, C. H., Proc. Soc. Exp. Biol. and Med., 1944, **57**, 19.

[3] Morgan, A. F., and Simms, H. D., *Science*, 1939, **89**, 565.

[4] Daft, F. S., and Sebrell, W. H., *U. S. Public Health Reports*, 1939, **54**, 2247.

[5] Ralli, E. P., and Graef, I., *Endocrinology*, 1943, **32**, 1.

[6] Deane, H. W., and McKibben, J. M., *Endocrinology*, 1946, **38**, 385.

TABLE I.
Supplements Added to 100 g Basal Diet (22% Vitamin-free Casein, 64% Sucrose, 9% Primex, and 5% NaCl-free Salt Mixture).

	Diet 100	Diet 1	Diet 14
Cod liver oil	1.9 ml	1.9 ml	1.9 ml
d, l-αtocopherol acetate	145 mg	.0 ’’	.0 ’’
Thiamine chloride	1.0 ’’	.3 mg	.3 mg
Pyridoxine chloride	1.0 ’’	.3 ’’	.3 ’’
Nicotinic acid	10 ’’	.0 ’’	.0 ’’
p-Aminobenzoic acid	30 ’’	.0 ’’	.0 ’’
Riboflavin	2.0 ’’	.9 ’’	.9 ’’
Inositol	5.0 ’’	.0 ’’	.0 ’’
Choline chloride	100 ’’	.0 ’’	.0 ’’
Biotin	.05 ’’	.0 ’’	.0 ’’
Pteroylglutamic acid	.20 ’’	.0 ’’	.0 ’’
Calcium pantothenate	1.0 ’’	.0 ’’	43.6 ’’

were used. They were fed one of the 3 diets whose composition is shown in Table I. Diet 1, the pantothenate deficient diet, and Diet 14, the high pantothenate diet, have been used in previous experiments reported from this laboratory.[5,7,8] Diet 100 is a modification of a diet used by Emerson.[9] The rats consumed 10-15 g of Diets 14 and 100, and 6-10 g of Diet 1 daily.

Experiment 1. The response to stress of normal rats on a complete diet containing an adequate[10] but minimal quantity of calcium pantothenate was studied. Rats 80 days of age were placed on Diet 100 *ad lib.* and 1% NaCl as drinking water for 2 weeks. At the end of this time they were subjected to swimming in large stone crocks filled up to 4 inches of the top with tap water at 25°C. The rats were obliged to swim for 25 minutes. Just before swimming and at 2, 4, and 6 hours afterwards, tail blood was taken for total leucocyte counts and for blood smears. The total leucocytes were counted in the usual manner; estimation of the per cent lymphocytes was based on differential counts of at least 200 cells. The white blood cell response was studied in rats fasted for 17 hours before swimming and in rats allowed food up to the time of swimming. All animals were provided with food after swimming.

Experiment 2. Male rats 30 days of age were placed on the pantothenate deficient diet (Diet 1) and were given 1% NaCl as drinking water. After 25-30 days on the deficient diet, the animals were swum under the same conditions as the rats in Experiment 1 and the response of the white blood cells was followed.

Experiment 3. After 30 days on the deficient diet the rats were transferred to Diet 14, which provided them with about 4 mg of calcium pantothenate daily. After 4-12 days on this diet the stress experiments were repeated and the response of the white blood cells observed.

Experiment 4. Normal rats on Diet 100 and pantothenate deficient rats on Diet 1 were given 4 mg ACTH[†] per 100 g body weight intraperitoneally. Immediately before the injection of ACTH and at 1, 2, 3, 4, 6 and 8 hours afterward the total leucocytes and percentage leucocytes were determined as before. The animals were not fasted for this experiment.

Results. The response of the white blood cells following swimming or ACTH in the rats on the various diets is shown graphically in Fig. 1. The figures on the left summarize experiments in which food was allowed up to the time of stress, and on the right the response following a period of fasting is shown. Each point represents the average of counts on 12-21 rats.

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[7] Ralli, E. P., *Endocrinology,* 1946, **39**, 225.

[8] Dumm, M. E., and Ralli, E. P., *Endocrinology,* 1948, **43**, 283.

[9] Emerson, G., personal communication, 1948.

[10] Unna, K., and Richards, G. V., *J. Nutrition,* 1942, **25**, 545.

[†] The ACTH used in these experiments was supplied by the courtesy of Dr. John Mote of Armour and Company.

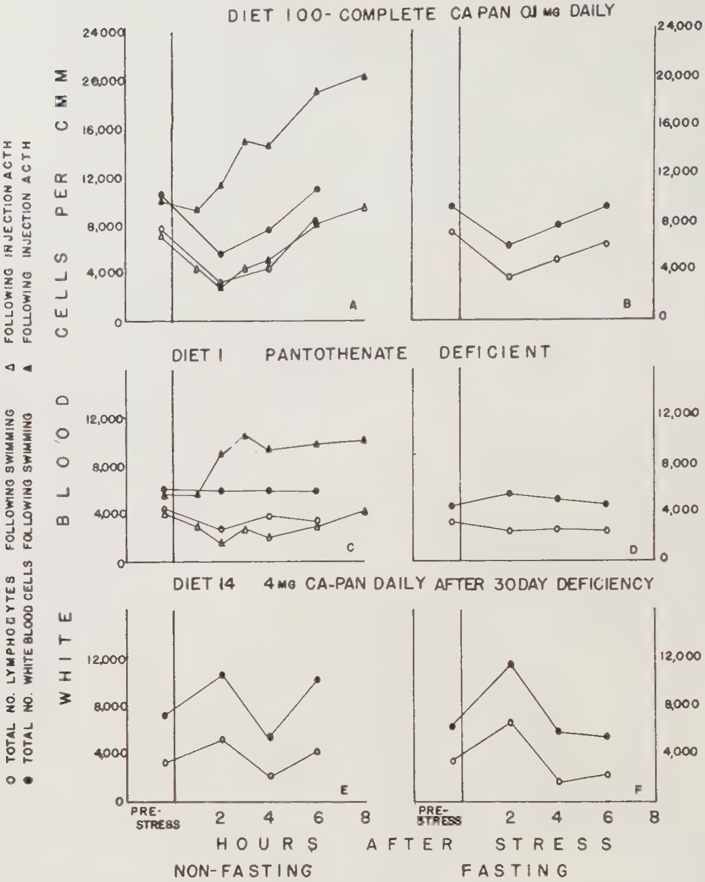

Fig. 1.

Effects of stress on response of white blood cells in rats on diets deficient in and supplemented with pantothenic acid.

The results of the experiments on normal rats maintained on Diet 100 are shown in Figure 1, A and B. This diet provided about 0.1 mg of calcium pantothenate daily. The experiments illustrate the marked lymphopenia which is known to occur when normal rats are subjected to stress.[1] The lymphocyte response was the same after swimming as after the injection of ACTH. Two hours after swimming or ACTH the lymphocyte counts had decreased to about half the initial values. After 6 hours the number of cells had returned to their initial values. The total leucocytes also decreased following swimming, but increased to about twice their initial value following ACTH. The results of the swimming experiment with fasted normal rats (Fig. 1, B) were similar to those in fed rats, although the initial counts were slightly lower.

The results in the pantothenic deficient rats are shown in Fig. 1, C and D. The total number of white blood cells and lymphocytes was lower in these rats than in the rats on the adequate diet. Both swimming and the injection of ACTH were followed in these animals by a small, but probably significant ($p < .05$), decrease in lymphocytes after 2 hours. The lymphocyte response was more pronounced after ACTH than after swimming. After ACTH the total number of white blood cells increased to about twice their original level but there was no significant change in the total number of white blood cells after swimming in the fed animals and only a small increase in the fasted rats.

Discussion. The data presented show that pantothenic acid can modify the response of the white blood cells in rats subjected either to a natural form of stress or to ACTH. In rats on normal diets both swimming and ACTH were followed by a significant decrease in the number of lymphocytes, occurring within 2 hours. In rats that had been fed a diet deficient in pantothenic acid for 3-4 weeks the lymphopenia following stress was largely abolished. Lipid depletion of the fascicular and reticular zones of the adrenal cortex has been reported after a period of pantothenic acid deficiency[5,6] and these are the zones probably responsible for the secretion of the corticosterone-like substances.[11] Dougherty and White[1] have shown that the injection of corticosterone in mice will produce lymphopenia. It may well be that the lipid depletion of the adrenal cortex produced by pantothenic acid deficiency was responsible for the decreased lymphocyte response to stress in the rats.

The addition of very large doses of calcium pantothenate to the diet of deficient rats restored the white blood cell response to stress. The nature of the response under these conditions was not that of the normal rat. The lymphopenia was delayed until the 4th hour after stress and was preceded by an increase in the lymphocytes at the 2nd hour. This occurred in both the fasted and non-fasted rats.

While general malnutrition is associated with adrenal cortical changes, it seems unlikely that this was an important factor in the results presented here. Deane *et al.*[6,12] have reported that neither pyridoxine nor riboflavin deficiency produced lipid depletion in the adrenal cortex of the rat and that inanition alone did not exhaust the adrenal as rapidly as a deficiency in either pantothenic acid or thiamine.

The decrease in the response of the white blood cells to stress in the animals on diets deficient in pantothenic acid is consistent with the changes in the adrenal cortex associated with the deficiency and is further evidence that pantothenic acid plays an important role in maintaining adrenal cortical function.

Summary. The responses of white blood cells and lymphocytes following swimming and following ACTH were compared in rats on a complete diet, in rats on a pantothenic acid deficient diet, and following swimming only in rats recovering from a period of pantothenic acid deficiency. A typical lymphopenia occurred 2 hours after either swimming or ACTH in rats on the complete diet. This response was partially abolished following either swimming or ACTH in the rats on the pantothenate deficient diet. In rats recovering from pantothenate deficiency, there was an increase in lymphocytes 2 hours after swimming followed by a decrease at 4 hours. These results are interpreted as a reflection of the changes in the adrenal cortex induced by pantothenic acid deficiency.

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[11] Deane, H. W., and Greep, R. O., *Am. J. Anat.*, 1946, **79**, 117.

[12] Deane, H. W., and Shaw, J. H., *J. Nutrition*, 1947, **34**, 1.

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17195. An Improved Method for the Microbiological Assay of Growth Factors on Paper Chromatograms.*

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The use of paper partition chromatography in the separation of microbial growth factors has recently been reported by Winsten and Eigen[1,2] and Cuthbertson and Smith.[3] These workers placed the developed chromatogram strips on nutrient agar seeded with the test organism. After incubation, zones of growth of the organism were noted along the site of each strip, indicating the presence of various growth factors. The relative concentration of these factors was estimated by the size of the zones.

The method of Winsten and Eigen[1] has been modified so that the chromatogram may be assayed both qualitatively and quantitatively for microbial growth factors using an improved technic. The improved method involves a direct test-tube microbiological assay of the developed chromatogram rather than the more tedious agar plate procedure. A description of the improved method, as used in the microbiological assay of paper chromatograms on which crystalline vitamin B_{12},† thymidine,† and injectable liver extract† were placed, is given in this report. *Lactobacillus leichmannii* (ATCC 4797) was used as the assay organism.

According to Shive, Ravel and Harding,[4] vitamin B_{12} and thymidine are interchangeable growth factors for *L. leichmannii* (ATCC 4797). Shive, Eakin, Harding, Ravel and Sutherland[5] have reported that thymidine may be present in amounts as high as 1% in some experimental injectable liver extracts. The presence of thymidine, therefore, may produce erroneous results in assaying liver preparations and other materials for vitamin B_{12}. By applying the procedure described in this paper, thymidine interference may be avoided.

Method. Whatman No. 1 filter paper (46 cm × 57 cm) was used in a chromatogram chamber similar to that described by Consden, Gordon and Martin.[6] A line was drawn across the paper 10 cm from the top edge. Along this line were placed small dots 3 cm apart. A known volume of the solution to be tested was placed on each dot with a microliter pipette using graded levels of from 1 to 10 microliters. In general each level was placed on from 2 to 4 dots. Evidence that the results could be duplicated was obtained by this means.

In no case was the spot of liquid allowed to spread more than 1 cm in diameter. Spreading was prevented by applying the liquid to the paper in portions of 3 microliters or less and allowing the liquid to evaporate between

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* This work was undertaken in cooperation with the Office of Naval Research, Navy Department, Washington, D.C., and was aided by grants from the Nutrition Foundation, New York City, the Cerophyl Laboratories, Kansas City, Mo.; Commercial Solvents Corporation, New York City; the Cooperative G.L.F. Exchange, Ithaca, N. Y., and the Western Condensing Co., San Francisco. The technical assistance of Betty F. Brown and Diana M. Cameron is acknowledged.

[1] Winsten, W. A., and Eigen, E., Proc. Soc. Exp. Biol. and Med., 1948, **67**, 513.

[2] Winsten, W. A., and Eigen, E., *J. Biol. Chem.*, 1949, **177**, 989.

[3] Cuthbertson, W. F. J., and Smith, E. Lester, *Biochem. J.*, 1949, **44**, No. 2 (*Proc. Biochem. Soc.*, v).

† The crystalline vitamin B_{12} used in this work was kindly supplied by Dr. E. Lester Smith of Glaxo Laboratories, England, and the thymidine by Dr. William Shive of the University of Texas. The injectable liver extract was Lilly's extract containing 15 U.S.P. units per ml.

[4] Shive, W., Ravel, J. M., and Harding, W. M., *J. Biol. Chem.*, 1948, **176**, 991.

[5] Shive, W., Eakin, R. E., Harding, W. M., Ravel, J. M., and Sutherland, J. E., *J. Amer. Chem. Soc.*, 1948, **70**, 2299.

[6] Consden, R., Gordon, A. H., and Martin, A. J. P., *Biochem. J.*, 1944, **38**, 224.

TABLE I.
Results of Assay of Paper Chromatograms Containing Injectable Liver Extract, Vitamin B_{12} and Thymidine.

Substance assayed	Amt. placed on paper	Location of growth by number of 2 cm section	Distance to solvent front in cm	Recovery in growth zone in %
Crystalline vitamin B_{12}	0.1 mγ	1	44.5	98.3
	0.2 ”	1	37.0	104.4
	0.3 ”	1	42.5	98.8
Liver extract, 15 units/ml	0.00015 unit	1	44.2	106.7
	0.0003 ”	1	43.5	100.9
	0.0015 ”	1	37.0	100.0
	0.0030 ”	1	37.0	100.0
Thymidine	0.5 γ	4–7	35.4	—*
	1.0 γ	4–8	35.4	—
	1.4 γ	4–8	35.4	—
	2.1 γ	4–8	34.8	—
Vitamin B_{12}	0.3 mγ	1	43.4	100.0
+ thymidine	0.2 γ	5–7		—
Liver extract	0.00015 unit	1	35.4	103.3
+ thymidine	0.5 γ	4–7		—
Liver extract	0.0045 unit	1	35.4	96.0
+ thymidine	1.4 γ	3–8		—

* Determination was omitted because sufficient thymidine was not available to prepare standard curves.

applications. An electric fan facilitated rapid evaporation. The spots were air dried and the paper was then placed in the chromatogram chamber with the upper edge immersed in the solvent trough.

The solvent used was n-butyl alcohol saturated with water. This was placed in the trough and also in each of two beakers at the bottom of the chamber to insure complete saturation of the chamber with the solvent vapors. The chromatographic procedure was carried out at room temperature. After 24 hours, or when the solvent had advanced approximately 40 cm, the paper was removed and suspended in a hood in front of a fan until all traces of solvent had disappeared.

The paper was then cut lengthwise in strips 3 cm wide so that each strip contained the material from one spot. These strips were then cut crosswise into sections 2 cm wide. The small sections of filter paper were each placed in test tubes, 10 ml of a newly developed medium[7] added and the tubes steamed 15 minutes. This was followed by inoculation with *L. leichmannii* (ATCC 4797) using a drop inoculator. Drop inoculation was found necessary since loop inoculation resulted in fraying of the filter paper which caused increased turbidity. After 16 hours incubation, growth was measured turbidimetrically in a Coleman Spectrophotometer set at 650 millimicrons.

When carrying out quantitative assays, the values for the standard curves were obtained by placing graded amounts of the standard solution of crystalline vitamin B_{12} on 2 x 3 cm sections of dried, butanol-treated filter paper using a microliter pipette and assaying these along with the developed chromatogram.

The presence of filter paper had no significant effect upon the growth of the organism. Pieces of filter paper ranging in size from 1 x 3 cm to 6 x 3 cm were tested and no appreciable differences in growth noted.

Results and Discussion. In Table I are given some typical results using the improved procedure in the assay of chromatograms for vitamin B_{12} and thymidine. The method was found to be sensitive to 0.1 millimicrograms of vitamin B_{12} present in the developed chromatogram. No measureable destruction of vitamin B_{12} was noted in the chromatographic procedure.

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[7] Peeler, H. T., Yacowitz, Harold, and Norris, L. C., unpublished results.

In the assay of the liver extract and crystalline vitamin B_{12} all the growth was confined to the first 2 cm section of the chromatograms. The remaining sections of the chromatogram failed to promote any growth response, indicating that the liver extract used in our experiments contained little, or no, thymidine or other growth factors. Interference from thymidine, therefore, did not occur when assaying the liver extract at a level of 0.0045 unit, or less, per tube.

In preliminary work it is necessary to assay each cross sectional piece of filter paper in the entire length of the chromatogram. Once the range of R_f‡ values of any substance has been accurately determined, it is only necessary to test the section known to contain that particular substance. For example, after separating thymidine from vitamin B_{12}, only the first 2 cm section of the chromatogram needs to be tested to obtain the vitamin B_{12} content of the substance being assayed.

The R_f value of vitamin B_{12} was found to be less than 0.04 while the large thymidine zone had an average R_f value of 0.33 and varied between 0.31 and 0.37. This thymidine R_f value differs from that reported by Hotchkiss[8] who obtained an R_f value of 0.51 using the same solvent but in the presence of gaseous ammonia. Winsten and Eigen[2] reported a double zone of growth for thymidine with the principal zone having an R_f value of 0.54 and a minor zone with an R_f value of 0.41. These latter workers were not able to state unequivocally which of these zones was due to thymidine.

The improved procedure has also been used successfully with the ascending solvent technic described by Williams and Kirby.[9] It appears applicable to the study of unidentified growth factors required by other microorganisms as well as *L. leichmannii* (ATCC 4797) and in the study of growth inhibitors and antibiotics.

Summary. A simple, quantitative method for the microbiological assay of filter paper chromatograms has been described. Some possible applications have also been pointed out.

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‡R_f (rate of flow) = $\frac{\text{Distance from starting line to center of growth zone}}{\text{Distance from starting line to solvent front}}$

[8] Hotchkiss, R. D., *J. Biol. Chem.*, 1948, **175**, 315.

[9] Williams, R. J., and Kirby, H., *Science*, 1948, **107**, 481.

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17196. Duodenal Ulcers Produced on a Diet Deficient in Pantothenic Acid.*

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It is well known that various nutritional essentials are concerned with the maintenance of normal epithelial tissue. As part of a program concerning nutritional deficiencies and gastrointestinal epithelium, we have reported the finding of marked antral gastritis on calcium deficient diets.[1] Similar, less marked and less constant lesions were also reported on thiamine deficiency. Under various conditions of inanition the fundic mucosa will show rather typical areas of hemorrhage.[2] Previously, many papers have appeared on the

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* This investigation was supported by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service.

[1] Zucker, T. F., Berg, B. N., and Zucker, L. M., *J. Nutrition*, 1945, **30**, 301.

[2] Zucker, T. F., Berg, B. N., and Zucker, L. M., *J. Nutrition*, 1945, **30**, 319.

involvement of nutritional deficiencies in the production of hyperplasia of the forestomach epithelium of rats.[3] In none of these cases, even when modifications were introduced to accentuate the process, have penetrating lesions been observed which could in the narrower sense of the word be designated as ulcers or which resembled typical ulcers as they occur spontaneously in man.

Further observations have shown that on a diet deficient in pantothenic acid penetrating ulcers are produced in rats. The diet contained all the known nutritional factors necessary for growth except pantothenic acid, and had the following composition per 100 g of diet: vitamin "free" casein (Labco) 18.0; cerelose 71.9; salt mixture 6.1 (Ca 1.0, P 0.55); cottonseed oil 2.0; celluflour 2.0. Incorporated in the oil were 10 mg alpha tocopherol and 1500 international units of vitamin A as carotene. The celluflour carried: thiamine HCl and pyridoxine HCl 1 mg each; riboflavin 2 mg; niacin 4 mg and 2-methylnaphthoquinone 0.5 mg.

Out of a larger group covering various age ranges, 45 animals have been autopsied: 23 older rats with an initial age averaging 394 (271-620) days and 22 younger rats starting at 42 to 105 days. The average experimental time for the older animals was 100 (67-140) days and for the younger ones 125 (87-185) days. As a rule the animals were autopsied when they showed severe weakness or an abrupt loss in weight.

Typical signs of pantothenic acid deficiency consisting of porphyrin deposit on the hair, degeneration, necrosis and hemorrhage in the adrenal cortex and fatty infiltration of the liver appeared in varying degree in all of the rats. Characteristic new findings were atrophy of the duodenal mucosa which was present in every animal, and duodenal ulcers which were encountered in 60% of the rats. The rest of the gastrointestinal tract was unaffected. Several hundred normal control rats and animals kept on diets deficient in other B factors failed to show similar changes in the duodenum.

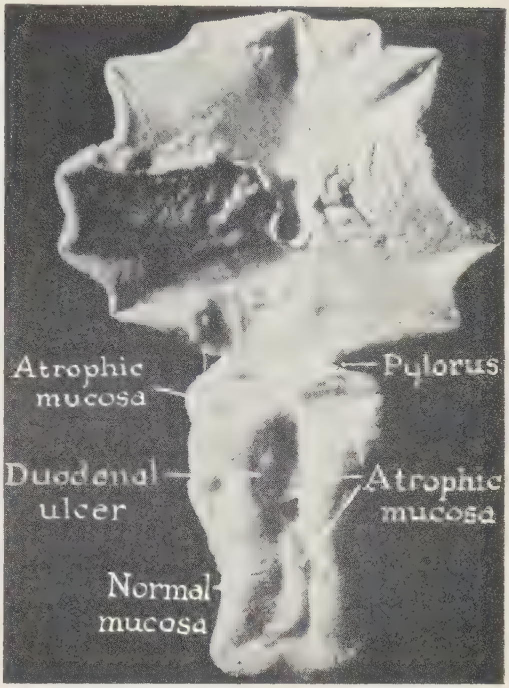

FIG. 1.
Large perforated duodenal ulcer. Base formed by connective tissue and adherent pancreas. Also shown, are areas of mucosal atrophy.

Areas of atrophy appeared as smooth depressions of various shapes and sizes. Sometimes the greater part of the surface was involved except for a few remaining islands of intact mucosa. Microscopically, villi were absent and were replaced by a flat layer of low cuboidal or squamous-like epithelium. Lesser degrees of atrophy were also noted. Here the villi were shortened or resembled simple tubular glands.

Ulcers have thus far been seen chiefly in atrophic mucosa. They occurred in 17 older rats and in 10 younger ones; in 17 instances multiple ulcers were present. Grossly and microscopically the lesions corresponded to the so-called acute and chronic ulcers of humans. Acute lesions extending for a variable distance through the different layers of the duodenal wall were found in all of the 27 animals. In 6 rats (5 belonging to the older group) coexisting large perforated defects sealed by dense connective tissue and adherent pancreas were also observed. (Fig. 1). A fibrinopurulent peritonitis due to perforation was encountered twice. Hemorrhage from eroded vessels in the fibrous base of ulcers was the cause of death in 2 animals.

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[3] Berg, B. N., *Am. J. Path.*, 1942, **18**, 49.

In swine, Wintrobe and associates have described atrophy and ulcers in the colon.[4] They also reviewed signs of pantothenic acid deficiency in other species. No duodenal lesions occurred among their reported findings.

Whether the experimentally produced ulcers will acquire significance for the study of human ulcers remains to be seen. The possibility that the latter condition is caused directly by dietary pantothenic acid deficiency is remote considering the exceedingly wide distribution of this factor. To base a working hypothesis on these observations it would be necessary to adduce evidence for abnormal paths of pantothenic acid metabolism. In rats, for instance, with adequate pantothenic acid in the diet but with prevention of intestinal synthesis of folic acid and biotin, a pantothenic acid deficiency in the tissues develops.[5] It is relieved by feeding folic acid and biotin. Orienting studies are under way to test out possibilities of pantothenic acid being involved in the human duodenal ulcer. In the meantime, however, the findings herein reported should be of value in elucidating the various steps of the pathological process which go into the formation of a duodenal ulcer, particularly the early stages which are seldom seen in human material.

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[4] Wintrobe, M. M., Follis, R. H., Jr., Alcayaga, R., Paulson, M., and Humphreys, S., *Bull. Johns Hopkins Hosp.*, 1943, **73**, 313.

[5] Wright, L. D., and Welch, A. D., *Science*, 1943, **97**, 426.

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17197. Neutralizing Antibody Against Viruses of the Encephalomyocarditis Group in the Sera of Wild Rats.

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The encephalomyocarditis (EMC),[1] MM,[2] Columbia-SK[3] and Mengo encephalomyelitis[4] viruses have been shown recently to be immunologically indistinguishable,[5,6] hence, they are apparently strains of the same agent. The viruses of the EMC family are now known to have a world-wide distribution, occurring in North America,[1-3] the Philippines,[7] and Africa,[4] but the natural history of the disease and sources of human infection are still obscure.

On at least two occasions viruses of the EMC group have been demonstrated to be pathogenic for man. Neutralizing antibodies against EMC virus have been shown to appear in the convalescent sera of a group of American soldiers who contracted a mild febrile illness colloquially designated as "Three-Day Fever"[7] in Manila in 1945-46. In addition, acute encephalomyelitis caused by Mengo virus occurred in a laboratory worker at Entebbe, Africa.[4]

We were led to consider the possibility that the wild rat might serve as a reservoir for viruses of the EMC type for a number of reasons:

Mice, hamsters and cotton rats are highly susceptible and succumb to even small doses

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[1] Helwig, F. C., and Schmidt, E. C. H., *Science*, 1945, **102**, 31.

[2] Jungeblut, C. W., and Dalldorf, G., *Am. J. Pub. Health*, 1943, **33**, 169.

[3] Jungeblut, C. W., and Sanders, M., *J. Exp. Med.*, 1940, **72**, 407.

[4] Dick, G. W. A., Best, A. M., Haddow, A. J., and Smithburn, K. C., *Lancet*, 1948, **2**, 286.

[5] Warren, J., Smadel, J. E., and Russ, S. B., *J. Immunol.*, 1949, in press.

[6] Dick, G. W. A., *J. Immunol.*, 1949, in press.

[7] Smadel, J. E., and Warren, J., *J. Clin. Invest.*, 1947, **26**, 1197.

TABLE I.
Neutralizing Antibody Against EMC Virus in Sera of Wild Rats.*

Locale	No. tested	Result† Negative	Positive	% positive
British Columbia	22	22	0	0
California	100	87	13	13.0
Florida	93	84	9	9.7
Georgia	57	51	6	10.5
Louisiana	9	9	0	0
Maryland	43	38	5	11.6
Mich. (Detroit)	11	11	0	0
Mississippi	49	6	43	87.0
New Mexico	6	6	0	0
North Carolina	2	2	0	0
South Carolina	21	21	0	0
Texas	23	16	7	30.5
Wash. (Seattle)	6	6	0	0

* Practically all the wild rats were *Rattus norvegicus;* some from California were *R. alexandrinus*, and all from South Carolina were cotton rats (*Sigmodon hispidus*).

† Sera containing sufficient antibody to protect against at least 100 minimal lethal doses of virus were considered positive, while those containing less than this amount were regarded as negative.

of virus injected peripherally.[2-5]

In contrast, the albino rat undergoes only an inapparent infection following a massive inoculum although considerable virus persists in the central nervous system[3,5,8] and the circulation.[9] The susceptibility of wild rats has not been determined but it was thought that it might be similar to the albino species.

One of the main foci of cases of Manila "Three-Day Fever" occurred in a camp located in a park which previously had been used by Japanese troops as a garbage dump. Although not mentioned in the original report of the outbreak, one patient whom we were able to interview recalled that rats were very numerous and cases of rat bite were occurring in this area.

Methods. The EMC, MM and Mengo viruses were maintained by intracerebral transfers in mice. The histories of the strains used in the present work were published in detail recently.[5] Rodent sera were collected in various regions of the country and shipped to this laboratory;* approximately half were refrigerated during transit. Neutralization tests were performed in 12-14 g albino Swiss mice using equal quantities of fresh infected mouse brain (appropriately diluted in distilled water) and undiluted serum. The virus-serum mixtures were incubated for 1 hour at 37.5°C after which 0.1 ml amounts of each mixture were inoculated intraperitoneally into each of 5 mice which were observed for 14 days. At the end of this time the neutralization index was calculated as the antilog of the difference of the LD 50% of the virus suspension in the control mixed with distilled water and in the test proper mixed with rat serum.

Experimental. Neutralization tests have been performed with sera from 442 wild rats (mainly *Rattus norvegicus* and *R. alexandrinus*) collected in various regions of the United States and Canada. Eighty-three of these had demonstrable antibody against EMC virus, *i.e.*, neutralized at least 100 M.L.D.'s of virus, and the majority of the positive sera possessed sufficient antibody to protect against 10,000 or more M.L.D.'s of virus.

The findings summarized in Table I show

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[8] Powell, H. M., Jamieson, W. A., and Culbertson, C. G., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 80.

[9] Warren, J., and Russ, S. B., unpublished experiments.

* This study was made possible through the generous cooperation of the following persons, who supplied us with rodent sera: Doctors G. C. Brown, D. Coburn, D. Davis, M. Frobisher, Jr., F. A. Humphreys, J. V. Irons, A. S. Lazarus, E. H. Lennette, L. Llewellyn, A. Morris, J. G. Rempel, V. R. Saurino, and M. Shaffer.

that the preponderance of sera examined came from rats caught in California, Florida, Georgia, Maryland and Mississippi, and that the proportion of positive sera among the rats from different regions varied considerably. For example, 43 of 49 specimens obtained from rural areas of Mississippi had antibody, whereas, only 6 of 57 from Georgia gave positive results. Certain counties within a state provided a greater proportion of positive sera than others. Thus, 11 of 44 samples from Orange County in southern California neutralized the virus, whereas, only 2 of 48 from San Diego County did so. Nine of the 93 sera examined from Florida gave positive results and 5 of these were among the five sera obtained from the town of Dania. It may be noted that it was in this area that the virus of encephalomyocarditis was originally recovered[1] from a chimpanzee held captive on an animal farm. Georgia supplied 6 positive sera and 5 of these were from rodents trapped in Wayne County. It would appear from the present data that the incidence of antibody against EMC virus is highest in rats caught in certain southern regions of the United States.

Twenty-two of the rat sera which were capable of neutralizing the EMC strain were also tested for their protective effect against the MM and Mengo strains. As was anticipated from earlier work the sera neutralized all 3 viruses to about the same extent.

EMC neutralization tests were performed on 69 sera obtained from various wild animals† which were caught in the same areas of California and Maryland that had yielded positive rat sera. (Table II). These included specimens from 4 gophers, 15 opossums, 9 rabbits, 18 squirrels, 2 chipmunks, 8 marmots, 2 meadow mice, 2 raccoons, 3 skunks and 6 domestic cats. None of these sera were capable of neutralizing even small amounts of virus, *i.e.,* 50 M.L.D.'s.

TABLE II.
Absence of Neutralizing Antibody Against EMC Virus in Sera of Rodents Other than the Rat.

Location	Species	No. tested	No. positive
Calif.	Gopher	4	0
	Opossum	13	0
	Rabbit	5	0
	Squirrel (*C. beecheyi*)	18	0
Md. (Patuxent)	Chipmunk	2	0
	Marmot	8	0
	Meadow mouse	2	0
	Opossum	2	0
	Rabbit	4	0
	Raccoon	2	0
	Skunk	3	0
	Domestic cat	6	0

Discussion. The EMC group of viruses appear to be ubiquitous, being widely distributed geographically and occurring in a number of host species. This should make one cautious in interpreting studies in which these viruses are apparently recovered from clinical material. Critical analysis of all the data is now required before one can assume that the agent found in passage animals bears an etiological relation to the disease in a given patient. Similarly, in view of present knowledge, "variant" strains encountered during serial transfer in animals such as the "high Lansing" mutant of poliomyelitis virus[8] should be thoroughly investigated for possible relation to the EMC group.

Summary. Sera from an appreciable number of 442 wild rats trapped in several widely separated areas of the United States contained neutralizing antibodies against the EMC group of viruses. The incidence of positive sera varied considerably in different geographic regions with the highest rate (87%) occurring in rats from the state of Mississippi.

Similar serological studies on other species of small wild animals failed to provide evidence of the occurrence among them of natural infection with the EMC viruses.

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† We are indebted to Drs. E. H. Lennette, State Department of Public Health, Berkeley, Calif., and J. A. Morris, Patuxent Research Refuge, Laurel, Md., for these sera.

17198. Relief from Pruritus Following upon Administration of Adenylic Acid.

ANTONIO ROTTINO. (Introduced by H. G. Albaum.)

From the Hodgkin's Disease Research Laboratory, Department of Medicine, St. Vincent's Hospital, New York City.*

In December, 1948, muscle adenylic acid was administered to a group of patients suffering from Hodgkin's disease in the hope that it might possibly have a beneficial influence upon their physical energy,[†] depletion of which is one of the disabling effects of this disease. Though the experiment proved a failure in this respect, a totally unexpected and gratifying result ensued: the only two patients of the group who had pruritus reported that this had completely disappeared. In an effort to establish whether or not this was mere coincidence the co-operation of colleagues[‡] was enlisted and medication extended to include persons afflicted with pruritus of diverse etiology.

In all, 36 patients were treated with the sodium salt of adenosine-5-monophosphate,[§] injected intramuscularly in a solution of 20 mg in 1 cc of water. The optimum dosage had to be determined by trial and error. Massive doses—100 mg hourly for 6 consecutive hours for a period of 11 days—was given to 4 patients (Nos. 1, 12, 28 and 35 in the table) and produced negative results on pruritis in all except case No. 28. Dosage of 20 mg hourly for 5 consecutive hours over a period of 3 days yielded fairly uniform results, including response by Case No. 1, who had not responded to the massive doses; Cases 12 and 35 were not retreated. Approximately half of the patients responded to the therapy within 24 hours, the others in from 2 to 7 days.

In 5 instances the pruritus was associated with diabetes (4 cases under insulin control and one as yet untreated), 9 with Hodgkin's disease, one with carcinoma of the ovary, one with dermatitis due to hair-dye sensitivity, 18 were idiopathic, and 2 occurred post partum. In almost every instance the pruritus was severe (inducing in some of the sufferers talk of suicide) and had previously been subjected to numerous therapies: salves, injections, x-ray, ultra violet rays, etc. Distribution was general in 20 instances, limited to anus or genitalia in 12 instances, to the legs, feet and hands in 4 instances. Skin of many of the patients was excoriated, in some instances severely so.

Results. Results were negative for 6 patients and positive for 30. There were 9 cases of complete subsidence, 14 of marked improvement, 5 of moderate improvement, and 2 of mild improvement. Not enough time has yet elapsed to make possible the assembling of statistics as to how enduring these results may be. Five patients (2, 28, 29, 30, 36) have remained free of recurrence or maintained the state of improvement from discontinuance of therapy to date, this period varying from one to four months; others suffered recurrence within 24 hours to one month. Three (16, 28, 29) who suffered recurrence and were retreated reacted as favorably to the remedication as to the initial medication.

The only toxic symptom—a sensation of inability to breathe freely—was short-lived and experienced by those receiving massive dosage (1, 12, 28, 35).

Discussion. Considering the fundamental importance of adenylic acid to biologic mechanisms such as glycolysis, the Kreb cycle, muscle contraction, etc., it is surprising that so little is known concerning the

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* Supported in part by grants from The National Cancer Institute of The U. S. Public Health Service, The American Cancer Society, and The Damon Runyon Memorial Fund.

[†] This was done at the suggestion of Dr. Kurt Stern, Brooklyn Polytechnic Institute.

[‡] Drs. Lloyd Craver, A. Susinno, Richard Kennedy, O. Canizares, A. Shapiro, J. Corr, W. Stankard, and F. Jost, to whom we express our sincere appreciation.

[§] The preparation used was "My-B-Den," made and supplied by Ernst K. Bischoff Co., Inc.

TABLE I.
Summary of Clinical Data on 36 Patients with Pruritus Who Were Treated with Adenylic Acid.

Pruritus						Results produced by Administration of My-B-Den		
Pt. No. and age	Sex	Etiology	Distribution	Severity	Duration	Relief	Appeared, day	Duration
1—41	F	Idiopathic	Vulva	4+	7 mo.	4+	2nd	1 wk*
2—35	F	”	”	4+	4 wk	4+	3rd	4 mo.*
3—30	F	”	”	4+	11 yr	0	—	—
4—56	F	”	”	4+	1 yr	4+	3rd	2 wk
5—60	F	”	”	4+	15 yr	3+	1st	2 wk*
6—74	M	”	Anus	4+	4 mo.	3+	1st	2 wk*
7—56	F	”	”	3+	3 wk	3+	1st	3 days
8—43	M	”	”	4+	8 yr	4+	2nd	1 wk*
9—42	M	”	”	4+	3 yr	3+	1st	1 wk*
10—39	M	”	”	4+	12 yr	3+	1st	1 wk*
11—56	M	”	Generalized	4+	6 mo.	4+	1st	3 wk*
12—35	F	”	”	4+	30 yr	0	—	—
13—87	F	”	”	4+	yrs	0	—	—
14—45	F	”	”	4+	6 mo.	2+	3rd	1 wk
15—43	F	”	”	4+	1 yr	2+	1st	No follow-up
16—22	M	”	”	3+	12 yr	3+	2nd	2 wk
17—67	M	”	”	4+	5 mo.	2+	3rd	2 wk
18—69	F	”	”	4+	5 yr	2+	4th	3 wk
19—30	F	Postpartum	”	4+	2 mo.	3+	1st	3 wk
20—32	F	”	”	4+	4 days	3+	7th	1 wk
21—62	F	Cancer	”	4+	6 mo.	3+	1st	3 wk*
22—64	F	Hair dye sens.	”	4+	10 days	0	—	—
23—65	M	Diabetes	Feet	4+	6 mo.	3+	1st	3 wk
24—86	F	”	Generalized	2+	yrs	1+	3rd	2 wk
25—60	F	”	Vulva	4+	4 mo.	2+	2nd	†
26—55	F	”	”	4+	9 mo.	3+	1st	2 wk*
27—67	F	”	Legs	2+	2 mo.	4+	3rd	3 mo.
28—39	M	Hodgkins	Generalized	4+	1 yr	4+	1st	5 wk
29—39	M	”	Hands	3+	1 yr	4+	6th	1 mo.*
30—49	M	”	Generalized	4+	1 wk	4+	3rd	1 mo.*
31—48	F	”	”	4+	6 yr	0	—	—
32—43	M	”	”	3+	1 mo.	3+	3rd	2 wk
33—52	F	”	”	4+	1½ yr	1+	1st	1 wk*
34—39	M	”	Legs	3+	7 mo.	3+	1st	1 wk*
35—40	F	”	Generalized	4+	3 yr	0	—	—
36—42	F	”	”	3+	1 mo.	3+	2nd	4 wk*

* Indicates patient was still free of pruritus at time this table was made.
† Only with medication. 20 mg hourly for five consecutive hours per day since May 7, ’49.
4+ indicates complete relief from pruritus.

clinical disorders resulting from pathologic-physiologic disturbance involving metabolism of adenylic acid and related substances.

We feel that the results which were obtained with adenylic acid are too consistent to be attributable to chance and that these results may be interpreted to mean that pruritus is one indication of adenylic acid deficiency. Adenylic acid has previously been used therapeutically[1-9] but so far as we know no one has pointed out the relationship between its administration and subsidence of pruritus. Beneficial effects on skin disorders have been

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[1] Bean, W. B., *Med. Clin. N. A.*, March 1943, **27**, 483.

[2] Carlström, B., and Olle Lövgren, *Acta Medica Scandinavica*, 1943, **115**, 568.

[3] Carlström, B., and Olle Lövgren, *Acta Medica Scandinavica*, 1942, **110**, 230.

[4] Dietrich, S., and Schwiegk, H., *Dtsche. med. Wchnschr.*, 1934, **60**, 967.

[5] Herbrand, W., *Dtsche. med. Wchnschr.*, 1937, **63**, 1841.

[6] Holyroyd, F. J., *West Virginia Med. J.*, 1940, **36**, 261.

[7] Rothmann, H., *Rev. Gastroenterol.*, 1942, **9**, 117.

[8] Spies, T. D., Bean, W. B., and Vilter, R. W., *Ann. Int. Med.*, 1940, **13**, 1616.

[9] Vilter, R. W., Bean, W. B., and Spies, T. D., *J. Lab. and Clin. Med.*, 1942, **27**, 527.

alluded to but not expanded upon.[3] We have not hitherto concerned ourselves particularly with this specialized field and can only state that several of our patients made voluntary mention of the fact that their skin had become softer and less dry and that excoriations due to scratching had healed upon subsidence of the pruritus some whose pruritus had not entirely disappeared found that scratching failed to produce the excoriation which had been the result previous to treatment.

Though there is much yet to be done clinically and in the laboratory before the full implications of adenylic acid administration to human beings can be completely evaluated, we feel that the results to date are sufficiently good to make broader experimentation desirable, and hence to warrant passing on our experiences to other workers.

Summary. Thirty-six patients suffering from pruritus of diverse etiology were treated with adenylic acid. In thirty instances there was a subsidence of the pruritus ranging from complete to mild. So far we have been able to find in the rather extensive literature no reference to the beneficial effect of adenylic acid upon pruritus.

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17199. Chemotherapy of Leukemia. IV. Effect of Folic Acid Derivatives on Transplanted Mouse Leukemia.*

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Since the first report of promising results in the clinical treatment of acute leukemia with 4-amino-pteroylglutamic acid,[1] this drug[2,3] and two other related compounds[3,4] have been reported to be active against certain strains of transplanted mouse leukemia. These clinical and experimental findings suggested the advisability of screening a large number of compounds related to pteroylglutamic acid (PGA) against transplanted mouse leukemia. This series included not only compounds closely related in structure to folic acid, but also pyrimidines, pteridines and purines. The results of the preliminary testing of 90 such compounds are herewith reported.

Method. The technic for evaluation of the chemotherapeutic activity of a given drug by means of its ability to prolong the survival time of mice with transmitted leukemia has been described previously.[3]

In a typical experiment, 240 mice of the inbred Akm stock were injected intraperitoneally with 0.1 cc of a saline suspension of leukemic spleen so diluted that 0.1 cc contained 1,000,000 cells. Leukemia Ak 4,[5] a relatively acute strain, was used in these par-

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† Fellow of The American Cancer Society, recommended by the Committee on Growth of The National Research Council.

[1] Farber, S., Diamond, L. K., Mercer, R. D., Sylvester, R. F., Jr., and Wolff, J. A., *New England J. Med.*, 1948, **238**, 787.

[2] Law, L., Abstract, Cancer Research, 1949, in press.

[3] Burchenal, J. H., Burchenal, J. R., Kushida, M. N., Johnston, S. F., and Williams, B. S., *Cancer*, 1949, **2**, 113.

[4] Burchenal, J. H., Bendich, A., Brown, G. B., Elion, G. B., Hitchings, G. H., Rhoads, C. P., and Stock, C. C., *Cancer*, 1949, **2**, 119.

[5] Burchenal, J. H., Biedler, J. L., Nutting, J., Stobbe, G. D., to be published.

TABLE I.
Compounds Showing Definite Chemotherapeutic Activity Against Leukemia Ak 4.

Compound	Dose mg/kg	Wt change (gr.)		Survival time (days)								% increase treated
				Untreated				Treated				
		Untreated†	Treated*	No. Mice	Range	Mean	S.D.	No. Mice	Range	Mean	S.D.	
4-Amino-N^{10}-methyl-pteroylglutamic acid	3	+1.5	+0.5	19	11-19	13.4	±1.78	10	28-39	30.1	±3.08	125
	3	+2.3	−0.7	20	10-16	12.4	±1.5	7	31-37	34.6	±1.84	179
	3	+1.9	+2.9	19	11-16	13.2	±1.57	8	26-45	31.9	±5.65	142
	3	+1.9	−1.2	20	11-18	13.5	±1.66	9	21-32	24.9	±3.57	84
	3	+3.7	+3.1	20	11-19	13.3	±1.78	8	30-43	34.2	±4.18	157
	3	+4.9	+2.4	20	10-15	12.7	±1.76	10	20-40	29.3	±5.44	131
	3	+2.3	+1.1	20	10-14	12.1	±1.26	17	20-36	27.0	±3.94	123
4-Amino-9-methyl pteroylglutamic acid	3	+1.7	+3.5	20	10-14	11.7	±1.12	6	19-29	24.5	±3.74	109
	4	+1.9	+1.8	20	11 18	13.5	±1.66	9	22-28	24.2	±2.57	79
	4	+2.9	+2.6	19	11-17	12.6	±1.70	7	19-43	29.3	±6.95	133
	4	+2.5	+5.5	20	10-18	11.7	±1.85	7	21-32	26.0	±4.29	122
	4	+3.3	+1.1	19	11-16	12.9	±1.47	6	33-41	37.1	±3.18	188
4-Amino-9,10 dimethyl pteroylglutamic acid	3	+1.7	+2.7	20	9 14	11.5	±1.24	10	22 34	29.5	±3.91	156
	5	+1.9	+1.0	20	11-18	13.5	±1.66	5	28-35	31.0	±2.68	129
	5	+2.5	+2.4	20	10-18	11.7	±1.85	5	32-38	34.2	±2.04	192
	5	+3.3	+0.9	19	11-16	12.9	±1.47	6	32-43	36.1	±3.58	180
	3	+1.7	−1.7	20	10-14	11.7	±1.12	6	28-42	34.2	±3.73	192
	3	+0.3	+0.5	16	10-14	12.2	±1.19	9	26-32	27.8	±1.81	128
2,6-Diaminopurine	90	+1.7	+0.9	20	10-16	12.0	±1.32	8	17-31	23.7	±4.44	97
	90	+0.8	+1.0	20	10-15	11.7	±1.74	9	19-26	22.5	±2.50	92
	90 × 2 75 × 8	−0.5	+1.4	20	10-14	11.3	±1.28	8	21-31	26.1	±3.85	131
	100	+2.3	−1.4	19	11-16	13.3	±1.87	10	20-40	28.6	±6.22	115
	90	+2.1	−0.5	19	9-16	10.7	±1.83	18	12-24	18.4	±3.94	72
	100	+1.0	−0.6	20	10-33	14.5	±4.50	9	20-31	24.4	±3.38	68

* Weight change calculated as the difference between the initial weight and that two weeks later.
† Weight change calculated as the difference between the initial weight and that one week later.
S.D. = Standard deviation.

ticular experiments. Forty-eight hours later, these mice were divided into comparable groups of 10 mice each (2 sets of untreated controls, one set of controls treated with a standard compound of known activity, 4-amino-N^{10}-methyl-pteroylglutamic acid, and 21 sets of mice treated with unknown compounds). Compounds were given intraperitoneally in maximum tolerated doses 3 times weekly for 10 doses. Water soluble compounds were dissolved in saline. Substances insoluble in water were usually suspended in 5% gum arabic in saline. The results of treatment with an unknown substance were compared with those obtained with the standard compound, 4-amino-N^{10}-methyl-PGA which has previously been shown to possess a high degree of chemotherapeutic activity[3] against Ak 4 leukemia. Maximum tolerated dosage was used throughout in an attempt to procure the maximum effect. The mice were observed for the development of leukemia and autopsied at death. If gross evidence of leukemia was not conclusive, microscopic sections were taken. The rationale behind the various steps of this technic has been outlined in previous reports.[6]

Results. The derivatives of pteroylglutamic acid which show a definite chemotherapeutic effect against Ak 4 leukemia are listed in Table I. The compounds included here are only those which show approximately a doubling of the survival time of the treated animals as compared with the untreated controls. Experiments in which these compounds have been evaluated are listed in detail. Further data on two compounds previously reported[3,4] (4-amino-N^{10}-methyl-PGA and 2,6-diaminopurine) are shown in Fig. 1. Table II includes those compounds which have shown a suggestive effect by increasing the average survival time approximately 50%. Table III lists the compounds which have shown no evidence of chemotherapeutic activity after at least one satisfactory test.

Discussion. In man, some leukemias do and some do not respond to antifolic therapy and, similarly, in the mouse not all strains of

TABLE II. Compounds Showing Slight to Moderate Chemotherapeutic Activity Against Leukemia Ak 4.

				Survival time (days)								
		Wt change (gr.)		Untreated				Treated				
Compound	Dose mg/kg	Untreated†	Treated	No. Mice	Range	Mean	S.D.	No. Mice	Range	Mean	S.D.	% increase treated
4-Amino-pteroyl-aspartic acid	30	+3.7	+4.6†	20	11-19	13.3	±1.78	10	15-25	18.1	±3.18	36
	30	+2.4	+2.7*	18	8-15	12.2	±1.61	8	17-28	21.5	±3.74	76
	30	+1.9	+2.4†	20	11-18	13.5	±1.66	10	14-32	18.4	±5.07	36
	30	+2.3	—0.9*	20	10-16	12.4	±1.50	9	15-26	20.2	±4.05	63
	30	+3.3	+0.6*	19	11-16	12.9	±1.47	9	15-53	25.5	±11.23	98
4-Amino-3′,5′-dibromo-pteroylglutamic acid‡	60	+1.5	+1.2*	20	9-16	12.5	±1.87	9	14-33	19.3	±5.4	54
	70	—0.9	+1.9†	19	10-22	13.5	±2.76	7	14-22	18.1	±2.54	34
4-Amino-pteroyl threonine	200	+0.3	+1.3†	15	10-14	12.2	±1.19	10	14-27	19.5	±4.46	60
	200	+3.5	+4.4*	19	10-13	11.0	±1.24	9	15-22	19.0	±1.88	73
4-Amino-pteroyl-glutamic acid	0.3	+3.7	+2.8*	20	11-19	13.3	±1.78	8	15-25	20.0	±3.24	50
	0.3	+3.3	+2.5*	19	11-16	12.9	±1.47	9	22-34	29.1	±3.24	126

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[6] Burchenal, J. H., Lester, R. A., Riley, J. B., and Rhoads, C. P., *Cancer*, 1948, **1**, 399.

TABLE III.
Compounds Showing No Evidence of Chemotherapeutic Activity Against Leukemia Ak 4.

Compound	Dose, mg/kg
Pyrimidines	
2-Amino-4-methylpyrimidine	750
2-Amino-4-(4′-arsonophenylamino)pyrimidine	15
2-Amino-4-methyl-5-acetylpyrimidine	64
2-Amino-4,5-dimethylpyrimidine	250
2-Amino-4,6-diacetylaminopyrimidine	175
2-Amino-4-hydroxy-5-(2′,4′-dichlorophenoxy)pyrimidine	1000
2-Amino-4-hydroxy-5,6-dimethylpyrimidine	250
2-Amino-4-hydroxy-5-p-chlorophenoxy-6-methylpyrimidine	1000
2-Amino-5-bromo-6-hydroxypyrimidine	250
2,4-Diamino-5-methylpyrimidine	75
2,4-Diamino-5-(2′, 4′-dichlorophenoxy)pyrimidine	250
2,4-Diamino-5,6-dimethylpyrimidine	35
2,4-Diamino-6-methylpyrimidine	125
2,4-Diamino-6-hydroxypyrimidine	450
2,5-Diamino-4,6-dihydroxypyrimidine	150
2,4-Dihydroxy-5-chloroacetamidopyrimidine	50
2,4-Dihydroxy-5,6-diaminopyrimidine	100
2,6-Dihydroxy-5-nitropyrimidine	150
2,6-Dihydroxy-5-bromopyrimidine	300
2,6-Dihydroxy-5-aminopyrimidine	250
2,6-Dihydroxy-4,5-diaminopyrimidine	15
2,4,6-Trihydroxypyrimidine	35
2,4,5,6-Tetrahydroxypyrimidine	250 × 2 125 × 7
2-Mercapto-4-hydroxypyrimidine	100
2-Mercapto-4-hydroxy-5-methylpyrimidine	1000
2-Mercapto-3-o-tolyl-4,6,6-trimethylpyrimidine	350
2,4,6-Trichloropyrimidine	15
2-Chloro-4-dimethylamino-6-methylpyrimidine	0.75
2-Methyl-4-hydroxy-5-ethoxymethylpyrimidine	750
1-Butyl-2-hendecyl-1,4,5,6-tetrahydropyrimidine	4
Hexahydropyrimidines	
1,3-Bis(1,3-dimethylbutyl)-5-nitro-5-methylhexahydropyrimidine	750
1,3-Bis(1,3-dimethylbutyl)-5-nitro-5-ethylhexahydropyrimidine	300
1,3-Bis(2-ethylhexyl)-5-amino-5-methylhexahydropyrimidine	35
1,3-Diisopropyl-5-amino-5-methylhexahydropyrimidine	125
1,3-Dibenzyl-5-nitro-5-methylhexahydropyrimidine	750
1,3-Dibenzyl-5-amino-5-methylhexahydropyrimidine	32
1,3-Di-p-tolyl-5-amino-5-methylhexahydropyrimidine	75
Pteridines	
2,4-Diaminopteridine	100
2,4-Diamino-6-methylpteridine	200 × 6 100 × 1
2,4-Diamino-7-methylpteridine	60
2,4-Diamino-6-p-carboxyanilinomethylpteridine*	125
2,4-Diamino-6-N-methyl-p-carboxyanilinomethylpteridine	125
2,4-Diamino-6,7-dimethylpteridine	50
2,4-Diamino-6,7-dihydroxypteridine	500
2,4-Diamino-6,7-diphenylpteridine	100
2,4-Diamino-6,7-dicarboxypteridine	250
2,4-Diamino-6,7-bis(4-aminophenyl)pteridine	500
2,4-Diamino-6,7-bis(p-sulfinomethylaminophenyl)pteridine	400
2,4-Diamino-5,7-dihydroxypyrimido(4,5-e)pteridine	300
2-Amino-4-hydroxypteridine	1000 × 1 667 × 3
2-Amino-4-hydroxy-6-methylpteridine	15
2-Amino-4,5,7-trihydroxypyrimido(4,5-e)pteridine	300
2,4,6,7-Tetrahydroxypteridine	10
2,4-Dihydroxy-6,7-dimethylpteridine	250
2,4-Dihydroxy-6,7-diphenylpteridine	125

Compounds directly related to pteroylglutamic acid	
Pteroylglutamic acid	60
Pteroyltriglutamic acid	400
N^{10}-methyl-pteroylglutamic acid	300
Sulfonamide analog of aminopterin‡	400
Pteroylglutamic acid γ-N,N-diethylamide†	200
Pteroylaspartic acid (d)	30
Pteroylaspartic acid (racemic)	30
4-Amino-pteroyl alanine	20
Glutamic acid derivatives	
N-(4-aminobenzoyl)-1(+)-glutamic acid	100
N-(4-aminobenzenesulfonyl)-1(+)-glutamic acid	500
Lumazines	
Lumazine	250
Dimethyl lumazine	325
Diphenyl lumazine	50
Purines	
Adenine	200
Guanine	500
Xanthine	500
Hypoxanthine	500
2,6-Diamino-7-methylpurine	250
Quinazolines	
2,4-Diaminoquinazoline	65
2-Methyl-4-hydroxyquinazoline	300
Quinoxalines	
2,3-Dihydroxyquinoxaline	70
2,3-Dichloroquinoxaline	30
Triazines	
4,6-Diamino-s-triazin-2-ol (Cyanurodiamide)	150
2,4-Diamino-6-(4-dicarboxymethylenethioarsenosoanilino)-s-triazine	30
Triazoles	
Benzotriazole	300
7-Amino-1-V-triazolo-(d)-pyrimidine	50
5-Amino-7-hydroxy-1-V-triazolo (d) pyrimidine	125

* This material is crude and nothing is yet known about the nature of the impurities.
† This compound is a crude product (analyzing about 11.3% pure).
‡ This compound is grossly impure.

transmitted leukemia are affected by this type of treatment.[2,3] A strain previously proven to be influenced by this type of therapy is, therefore, essential to such a screening program. The sole diet of the mice during the experiment consisted of Purina Laboratory Chow of an unknown, but presumably fairly constant pteroylglutamic acid content. No further measures for controlling the intake of the vitamin were attempted. Since the activity of various derivatives as anti-metabolites vary markedly,[7] it is quite possible, therefore, that if the anti-leukemic effect is related to the anti-folic activity, certain less effective antagonists of pteroylglutamic acid may have been missed by this lack of dietary control.

All compounds in these studies which showed a definite chemotherapeutic effect were related in that there were amino groups in the 2 and 4 positions of the pteridine ring or in the analogous configuration in 2,6-diaminopurine. The importance of this particular amino substitution of the pteridine nucleus has been demonstrated by Daniel in her studies on the anti-bacterial action of the pteridines.[8] Hitchings reported that, in studies of a large number of pyrimidines, an

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[7] Smith, J. M., Jr., Cosulich, D. B., Hultquist, M. E., and Seegar, D. R., *Tr. New York Acad. Sc.*, 1948, **10**, 82.

[8] Daniel, L. J., Norris, L. C., Scott, M. L., and Heuser, G. F., *J. Biol. Chem.*, 1947, **169**, 689.

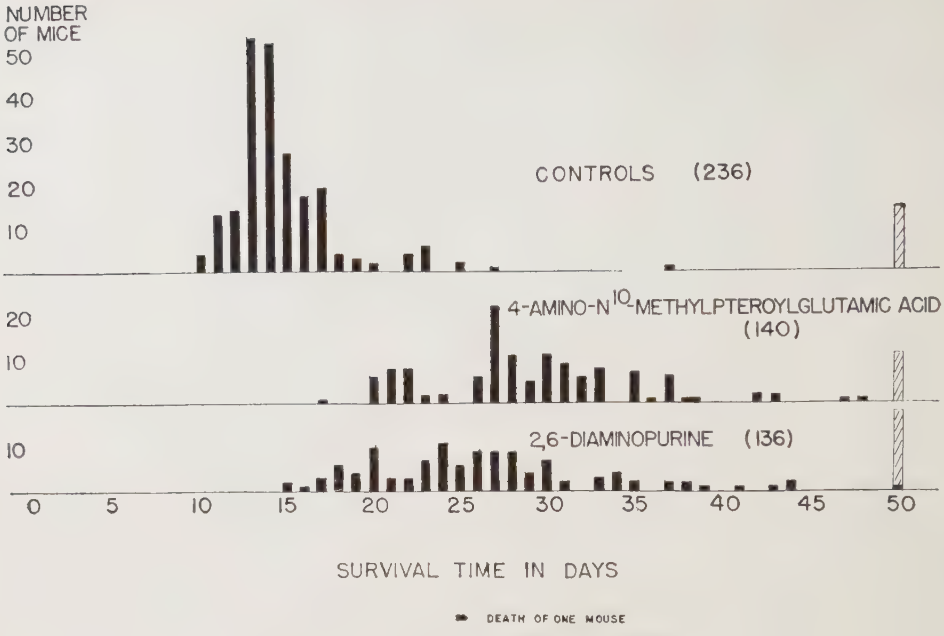

FIG. 1.

inhibition of growth of *L. casei* with PGA in the absence of purine was a property of nearly all 2,4-diamino-pyrimidines and their condensed systems.[9]

Despite the fact that certain simple pyrimidines[10] and pteridines[8] are antagonists of PGA in the metabolism of bacteria, it is of interest to note that all such compounds which were tested against Ak 4 leukemia were without definite effect. The addition of a para amino-benzoic acid moiety to the 2,4-diamino pteridines in 4-aminopteroic acid (2,4-diamino-6-p-carboxyanilinomethylpteridine) and in 4-amino-N^{10}-methyl-pteroic acid (2,4-diamino-6 - N-methyl-p-carboxyanilinomethylpteridine) did not increase activity. With the exception of 2,6-diaminopurine, the 2,4-diamino configuration of the pyrimidine ring was effective only when it was a portion of a larger molecule consisting of a pteridine, a para amino-benzoic acid, and an α-amino acid. 4-amino derivatives containing the α-amino acids, glutamic, aspartic or threonine, possessed chemotherapeutic activity, but 4-amino-pteroyl alanine was inactive.

Summary. 1. Ninety compounds related to pteroylglutamic acid have been tested for chemotherapeutic effect against transmitted leukemia Ak 4 in mice.

2. Eighty-two of these compounds showed no chemotherapeutic effect by this particular technic.

3. Four showed slight to moderate effect.

4. Four compounds, 4-amino-N^{10}-methyl-pteroylglutamic acid, 4-amino-9-methyl-pteroylglutamic acid, 4-amino-9,10-dimethyl-pteroylglutamic acid, and 2,6-diaminopurine have definite chemotherapeutic activity as demonstrated by approximately doubling the average survival time of the mice treated with these compounds.

5. The occurrence of an amino substitution in the second and fourth positions of the pyrimidine ring in all these active compounds has been noted.

We wish to acknowledge at this time the generosity of the following groups in supplying the compounds used in these studies: Dr. C. K. Cain, Department of Chemistry, Cornell University; Calco Chemical Company; Commercial Solvents;

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[9] Hitchings, G. H., Elion, G. B., Vander Werff, H., and Falco, E. A., *J. Biol. Chem.*, 1948, **174**, 765.

[10] Hitchings, G. H., Falco, E. A., Sherwood, M. B., *Science*, 1945, **102**, 251.

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17200. A Readily Soluble form of P. B. P. for Use as a Routine Diagnostic Test.

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We reported the preparation of a purified protein antigen from *Brucella*,[1] made by following a modification of Seibert's method for the preparation of PPD (purified protein derivative from tuberculin). This protein substance was a fine, light brown powder of fairly constant chemical composition, not completely soluble in water but easily dissolved by the addition of a few drops of 0.1 N alkali. The solution could be neutralized with 0.1 N HCl and remained clear. This preparation was used by us, as well as by other investigators, for the study of cutaneous hypersensitiveness to *Brucella*. As workers may not have laboratory facilities, we have devised a method whereby the PBP (purified brucella protein) is put up in vial form and in just the right amount for skin testing. By adding a measured quantity of sterile saline buffered solution, the PBP is ready for use.

To prepare the PBP in this way, a weighed amount of the protein antigen is dissolved, as indicated above, to make a concentration of one mg per cc. This solution is dialized through cellulose tubing (Visking Corp.) in cold water for 24 hours. Any impurity that may separate is removed by centrifugation. Then 50 mg of Beta lactose per cc of the PBP solution are dissolved. The resulting liquid should be completely clear. One half cc of this solution, containing 0.5 mg of the *Brucella* protein extract, are bottled in glass vials (about 8 ml capacity) of the type ordinarily used for vaccines, and the contents dried by lyophilization. The lactose has no deleterious effect on the PBP; does not influence the allergic reaction, and is used only to give bulk to the product after drying, since the amount deposited in each vial is infinitesimal.

For skin testing, 5 ml of the sterile diluting fluid are added to each vial, by means of a sterile syringe and needle through rubber stoppers of the vial, and shaken for 2 or 3 minutes to dissolve their contents. The diluting fluid is the same used for Seibert's PPD[2] and is a buffered saline liquid made from the following solutions:

KH_2PO_4: 9.078 g dissolved in 1000 cc of distilled water

$Na_2HPO_4 \cdot 2H_2O$: 11.876 g dissolved in 1000 cc of distilled water

Two parts of the solution of the potassium salt are mixed with 8 of the sodium salt, and 0.25% phenol is added.

Each vial contains enough PBP for 50 tests. The tests are performed by injecting 0.1 ml of the diluted protein product with a tuberculin type syringe and a 26-gauge needle into the skin over the flexor surface of the forearm. The site of the injection is examined 48 hours later. Subjects who react positively show a pronounced erythema and edematous induration. Negative cases show no changes.

This preparation was tested on 25 individuals supposed to have been in contact with infected material. Two of them were veterinarians and 2, their assistants. The rest were milkers and dairy workers. Of this group 8 gave positive and 17, negative.

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[1] Morales-Otero, P., and González, Luis M., Proc. Soc. Exp. Biol. and Med., 1938, **38**, 703.

[2] Seibert, F. B., *et al.*, *Am. Rev. Tuberculosis*, 1934, **30**, 707.

As a further check of the specificity of this lyophilized form of PBP, 10 patients from the University Hospital, who had been diagnosed as brucellosis cases and who had previously given a positive skin test with the old form of PBP, were again tested with this readily soluble form. All gave a positive skin reaction.

For negative control group, 20 healthy subjects, selected at random from the general population and with no history of brucellosis or symptoms usually associated with this condition, were also tested; all were negative to the lyophilized PBP.

Summary. A purified brucella protein, subjected to further purification and presented in a soluble lyophilized form, has been described. Weighed amounts are placed in glass vials, which, after the addition of a measured quantity of sterile saline buffered solution, produces a product ready for use. This procedure facilitates the use of the product in epidemiological investigation and as a routine diagnostic test.

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17201. Effects of Bone Extracts Injected into the Mouse Testis.*

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Bone will develop from bone marrow when it is transplanted under certain conditions. This subject has recently been reviewed by Levander[1] and Pfeiffer.[2] However, these two authors differ markedly in their interpretation of how bone is formed from the marrow transplant. Levander[1] has reported that when bone marrow is transplanted, all of the grafted cells disintegrate and mesenchymal cells from the surrounding tissue soon migrate into the graft and are transformed into chondroblasts and osteoblasts which form cartilage and bone. Pfeiffer[2] agrees with Levander that the hematogenic and myelogenic cells disappear from the graft but believes that when bone forms, the marrow reticulum cells of the graft survive and these cells develop into osteoblasts just as they do in the marrow cavity when medullary bone forms there as described by Bloom, Bloom and McLean[3] in the pigeon.

The evidence in the literature[4-7] that there is an extractable osteogenic substance would tend to support Levander's hypothesis. However, it has been questioned whether such a substance exists, and evidence that injury is the primary factor in causing bone to form when bone extracts are injected has been advanced by Heinen.[8] Apparently mesenchymal cells transform into osteoblasts in either case. It therefore becomes important to test whether the mesenchymal cells present in the area where bone developed in marrow grafts would produce bone under the same or greater injury than resulted from grafting, plus the extract from a similar or larger piece of marrow than was grafted.

Materials and methods. Mice of the Strong[9] A strain were used in this experiment because it has been shown that bone will develop in marrow grafts in the anterior chamber of the eye and in the testes of mice of

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* This investigation was aided by grants from The Anna Fuller Fund, the National Cancer Institute (U.S.P.H.S.), and the James Hudson Brown Fund.

[1] Levander, G., *Acta Chir. Scand.*, 1940, **83**, 545.

[2] Pfeiffer, C. A., *Anat. Rec.*, 1948, **102**, 225.

[3] Bloom, W., Bloom, M. A., and McLean, F. C., *Anat. Rec.*, 1941, **81**, 443.

[4] Annersten, S., *Acta Chir. Scandinav.* (Suppl. 60), 1940, **84**, 1.

[5] Annersten, S., *Chirurg.*, 1941, **13**, 76.

[6] Bertelsen, A., *Acta Orthopaedica Scandinavica*, 1944, **15**, 139.

[7] Levander, G., and Willistaedt, H., *Nature*, 1946, **157**, 587.

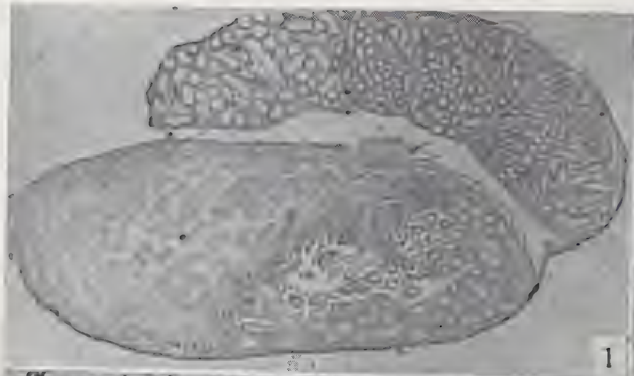
[8] Heinen, J. H., personal communication, 1948.

[9] Strong, L. C., *J. Hered.*, 1936, **27**, 21.

this strain.[2] Of these two sites, the testis was used since it contains numerous primitive mesenchymal cells. It nicely delimits the area affected, and it does not contain or allow any contact with the type of connective tissue associated with ligaments or bone attachments. The bone extract was made according to the procedure of Annersten,[5] with slight modification to adapt it to the mouse. All of the long bones, the pelves, the shoulder girdles, the calvarium and any other bones that could be readily freed from the surrounding tissue were removed from 6 or 7 mice. These bones, weighing 5 to 8 g were ground with a mortar and pestle and extracted with 15 to 20 cc of HCl-alcohol (100 cc of 95% alcohol and 5 cc of 0.1N HCl). The ground bone was placed in a glass stoppered tube. The alcohol was added and stirred. The mixture was then placed in a refrigerator at +1°C for 48 hours and stirred frequently. It was then centrifuged. The supernatant liquid was placed in an appropriate container and evaporated to one-quarter its former volume by passing a stream of warm air over it. The extract became opalescent and a fine lipid film formed around the edges of the surface. No attempt was made to analyze the content of this extract. However, Annersten[4] gives a report on the analysis of his extracts of rabbit bones. The solution was next diluted with an equal volume of isotonic salt solution and injected immediately. If injections were not to be made at once, the dilution was not done until just before injection. The extract was stored in the refrigerator at +1°C.

The extract was injected by means of a 1 cc tuberculin syringe equipped with a 25 gauge needle. The mice were anesthetized, and the left testis was exteriorized through a suprapubic midline incision. The testis was grasped by the gubernaculum, and the hypodermic needle inserted at the posterior pole of the testis, parallel to its long axis. As much extract as could be retained (about 0.05 cc) was injected. If too much solution was injected, the internal pressure caused herniation of the seminal tubules at the point of entrance of the hypodermic needle. The testis was then returned to the abdominal cavity and the incision sutured. Thirty-three male mice were used. The animals were autopsied at 15 days, and at 3, 4, 5 and 6 weeks after the extract was injected. The testes were preserved in Bouin's fluid, sectioned in paraffin (7 μ) and stained with Harris' hematoxylin and triosin.

Observations. In gross appearance the treated testes are somewhat less than 2/3 their normal volume. If the seminal tubules herniate, or some of the acid-alcohol extract leaks out, there is a tendency for the testis to adhere to surrounding tissue. Adhesion usually occurs in the open inguinal canal or in the region around the abdominal incision. The area that is affected by the extract can be readily seen in gross observation, since the tubules appear as chalky white structures showing through the tunica albuginea. The remainder of the testis is the normal pinkish gray color without the tubular nature of the testis being apparent. The extract seems to fix the tissue somewhat in about 2/3 of the testis (Fig. 1). At 2 weeks there are no lumina in the tubules of the treated area. The cell membranes are no longer apparent, and the coalescence of the cells produces a homogeneous light pink-staining granular mass. Apparently the nuclei of the basal layers of cells disappear, but the chromatin material of the spermatids and spermatozoa is grouped concentrically at the centers of the tubules. Nuclear membranes are not seen, yet the chromatin material is very dense and compact, without evident coalescence, and retains its hematoxylin-staining affinity. The connective tissue forming the tubule walls appears desiccated, but otherwise does not show much alteration. The interstitial tissue is composed of connective tissue, Leydig cells and occasional macrophages and lymphocytes. The cytoplasm of the connective tissue cells and Leydig cells takes the same light pink stain as does the material within the tubules. Some of the Leydig cells have the characteristic interstitial cell nuclei, while in others the nuclei are becoming pycnotic. The nuclei of the fibroblasts are in most instances pycnotic. Lymphocytes are not yet numerous, and the blood vessels show only slight evidences of future hyalinization. Macrophages are only occasionally seen.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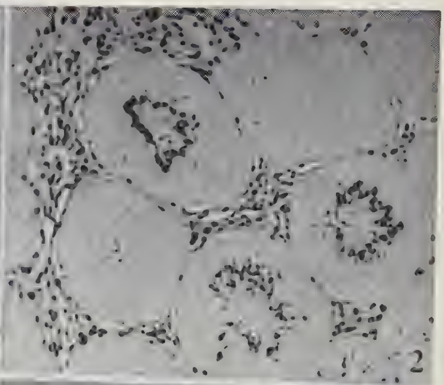
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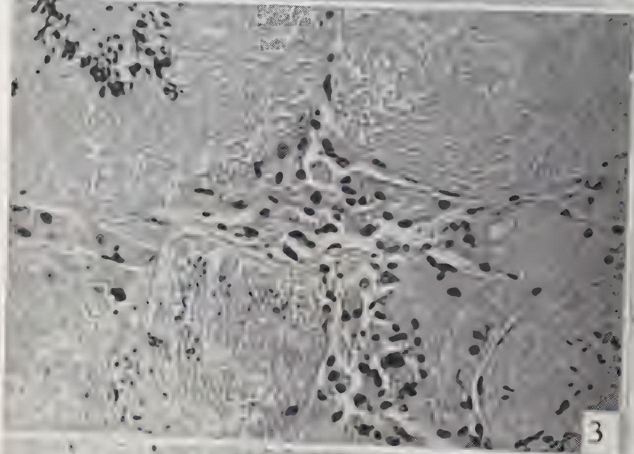
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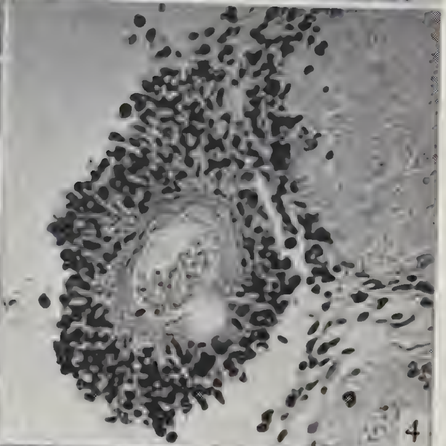
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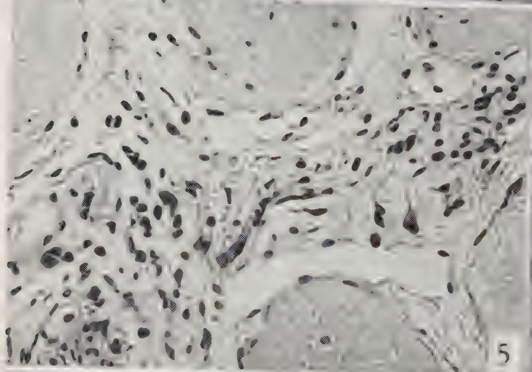
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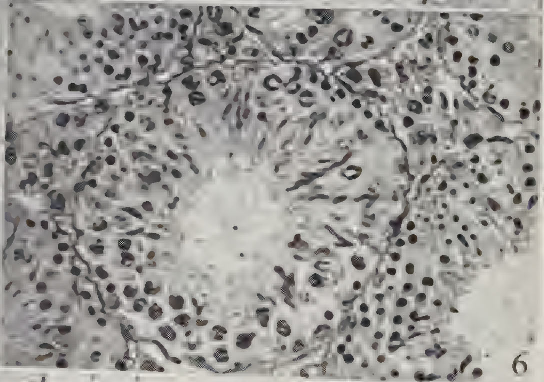
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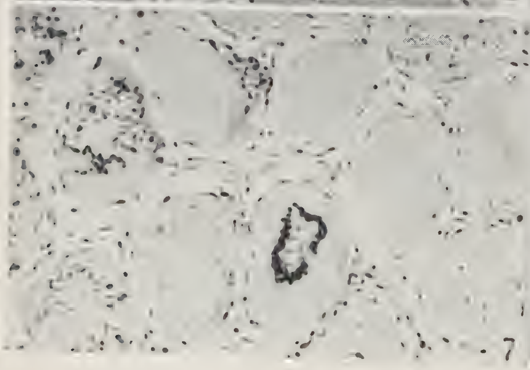
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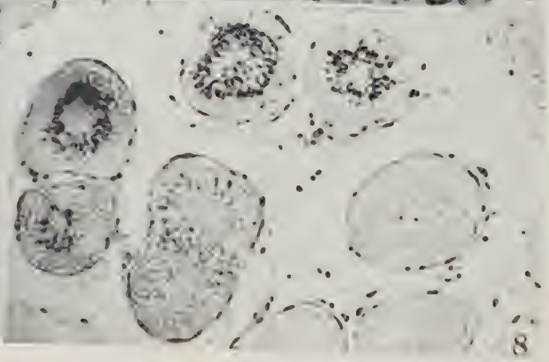
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All tissues were fixed in Bouin's fluid, sectioned at 7 μ and stained with Harris' hematoxylin and triosin.

FIG. 1. Longitudinal section through entire testis and epididymis following injection of 0.05 cc of an acid alcoholic extract of bone into the testis. The portion to the left is the treated area and that to the right is the normal testicular tissue. Note the sharp dividing line between the infarct and the normal area. × 12.

FIG. 2. A section of the treated area of a testis 20 days after injection of an acid alcoholic extract of bone. This section is near the normal part of the testis and shows the predominance of fibroblastic elements in the interstitial tissue of this area and the disappearance of all normal cellular structure in the seminiferous tubules, except for the darkly staining chromatin material of the spermatids and spermatocytes which is arranged concentrically in the center of the tubules. × 110.

FIG. 3. Same testis as in Fig. 2. A section near the center of the treated area. Note the rather normal appearing Leydig cell between the 2 tubules at the extreme right and the numerous cells in the interstitial tissue, some of which may be altered Leydig cells but most of which are undoubtedly macrophages. See also altered protoplasm, lack of chromatin material and even, faintly staining appearance of all spermatogenic cells. The chromatin material of the spermatids and spermatozoa still stains very strongly, however. × 220.

FIG. 4. A cross section of an arteriole at periphery of infarct in a testis 20 days after injection of bone extract, showing marked alteration of these vessels and presence of numerous plasma cells and lymphocytes around them. The remaining interstitial tissue appears greatly dehydrated but not otherwise much altered. × 220.

FIG. 5. Interstitial tissue 4 weeks after injection of bone extract. Note little change in the cells. They are extremely pycnotic, and the fibers appear denser. The small blood vessels show none of the changes seen at periphery. All of tissue has been killed and fixed by the alcohol and remains in this condition with no evidence of removal of dead tissue. × 220.

FIG. 6. The normal portion of a testis that had received acid alcoholic extract of bone 6 weeks before. The treated area of the same testis is shown in Fig. 8. × 220.

FIG. 7. Treated area of a testis 5 weeks after injection of bone extract. There is little change from earlier stages except that there is more interstitial edema. The tubules are smaller and there are possibly fewer chromatin remnants of the spermatids and spermatozoa in the tubules. × 110.

FIG. 8. Treated area from the same testis as shown in Fig. 6. It is similar to Fig. 7 except for the greater interstitial edema. At 6 weeks there is no evidence that the killed and preserved cells are being cleared out of the area. × 110.

At 3 weeks the contents of the seminiferous tubules are essentially the same as at 2 weeks (Fig. 2). The protoplasm is, however, somewhat more homogeneous. In the intertubular spaces there are fewer cells with definitive interstitial cell nuclei, but cells with pycnotic nuclei which might formerly have been interstitial cells of Leydig are fairly numerous (Fig. 3). They are, however, almost completely absent from the center of the treated area. The fibroblasts show little if any change from that seen at 2 weeks. A few polymorphonucleated cells are present at the periphery of the treated area. Also in this region are a few lymphocytes. The vascular condition has changed very little from that at 2 weeks. Hyalinization of the blood vessels is beginning at the periphery, and lymphocytes are becoming more numerous around these blood vessels (Fig. 4). At the dividing line between the treated and normal areas an occasional seminiferous tubule shows the characteristic clumping of the spermatocytes that occurs when the seminiferous tubules are damaged. This type of degeneration in the tubules is never seen except at the periphery of the treated area, and is secondary to the primary damage.

By 4 weeks the tubules are further shrunken in size. The protoplasm appears to be more homogeneous. There is less chromatin material visible, and many cross sections of tubules show none at all. There is much more intertubular edema, although this varies considerably from animal to animal. The nuclei of the fibroblasts are quite pycnotic (Fig. 5), and only rarely can a cell be found which could have been an interstitial cell of Leydig, even in the peripheral zone of the affected area. There is a somewhat marked hyalinization of the blood vessels, but it is only the vessels at the periphery of the treated area that show this response. Lymphocytes are much more numerous, especially near the blood vessels, and plasma cells are now present. Macrophages are not noticeable at this time. The amount of connective tissue in the interstitial spaces is about normal, but it appears dehydrated, and the large empty spaces due to further shrinkage of the tubules make it appear less abundant.

From the fourth to the sixth week the changes already described become more extreme (Fig. 7 and 8). There is further shrinkage of the tubules and disappearance of chromatin material, although certain tubules resemble those at 2 or 3 weeks, except for the smaller size. Usually only connective tissue which has been coagulated by the injected alcohol is seen in the intertubular spaces. The fibers often show evidence of disintegration. The spaces between the tubules, and between the tubules and connective tissue become greater. Apparently the shrinkage of the tubules is much greater than that of the connective tissue. In the central area only an occasional cell that may be a degenerating interstitial cell or a macrophage is seen. If these are living macrophages, there is no evidence of their phagocytic activity.

The vascular changes seen in the treated testes seem to be associated with the peripheral area, and the presence of lymphocytes and plasma cells is limited primarily to this region. Presumably the lymphocytes come in from the blood stream, but the plasma cells may possibly be formed in the area. There is no evidence whether the plasma cells arise from lymphocytes or not. They are, however, limited to the same areas. There seems to be no evidence of undamaged fibroblasts or primitive mesenchymal cells in the treated area. The vascular and lymphocyte response in the peripheral region occurs whether it is adjacent to normal seminiferous tubules or to the tunica albuginea. The tubules in the area outside of that exposed to the extract are essentially normal (Fig. 6) except for a slightly cryptorchid appearance which may be due to the fact that the testis is only about 2/3 its normal size and probably has resided in the abdominal cavity most of the time. The interstitial tissue and Leydig cells appear to be increased somewhat in this area. This is probably due to the reduced tubular volume.

Discussion. The fact that bone did not develop in the mouse testis when acid alcoholic extract of bone was injected, even though the amount of extract injected represented a volume of marrow many times that which was transplanted to the mouse testis and which formed bone,[2] strongly suggests that the marrow graft contributes something besides a possible extractable osteogenic substance, presumably the growing marrow reticulum cells, which it seems probable become osteoblasts and form bone.

In the literature on the transplantation of bone marrow the following facts are evident: 1. Autoplastic transplants of bone marrow are fairly successful in forming bone.[1,10–14] 2. Homoplastic transplants are seldom successful[10,11,15,16] and then only in young animals, and with large amounts of marrow.[1,17] 3. In inbred strains of mice implantation of tiny fragments of marrow cause the development of bone.[2] 4. The degree of resistance to grafting which is present in the grafting site is of extreme importance; where resistance is high, bone has not formed when marrow is transplanted.[2] All of these conditions are related to the ability of grafted cells to grow in their new environment. It seems, therefore, that bone develops in marrow transplants only under conditions that are favorable for transplantation. Since all workers agree that the myelogenic and hematogenic cells do not survive grafting, it must be that the marrow reticulum cells do survive as suggested by Pfeiffer[2] and are the cells which Levander[1] believes are mesenchymal cells which have migrated into the area.

While it seems quite clear from the above evidence that marrow reticulum cells form

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[10] Baikow, A., *Centralbl. f. d. Med. Wiss.*, 1870, **8**, 371.

[11] Bruns, P., *Arch. f. klin. Chir.*, 1881, **26**, 661.

[12] Bull, Ch. R., *Experimentelle Studien über Knochentransplantation und Knochenregeneration*, 1928, J. Dybwad, Oslo.

[13] Chiari, O. M., *Münch. Med. Woch.*, 1912, **59**, 2502.

[14] Miyauchi, K., *Arch. f. klin. Chir.*, 1914, **106**, 273.

[15] Ollier, L., Traité experimentale et clinique de la régénération des os et de la production artificielle du tissu osseux. T. I. partie experimentale, 1867, Victor Masson et Fils, Paris.

[16] Maas, H., *Arch. f. klin. Chir.*, 1887, **20**, 708.

[17] Goujon, E., *J. de L'Anat. et de La Physiol.*, 1869, **6**, 399.

bone when bone marrow is transplanted, just as they have been shown to do in their normal position,[3] the evidence is equally convincing that bone is caused to form in many areas where marrow reticulum cells are not present. The osteoblasts and chondroblasts in the latter areas must come from the primitive mesenchymal cells. However, in spite of the obvious similarity of the primitive mesenchymal cells and the marrow reticulum cells new problems become evident. The injection of an acid alcoholic extract of bone into muscle has been reported to cause the primitive mesenchymal cells to form bone,[1,4-6] yet the testis of the mouse contains large numbers of primitive mesenchymal cells which failed to respond to acid alcoholic extract of bone. In the present experiment all the tissue in the treated area is killed. It represents a subtotal infarction, and apparently the damaged tissue is gradually removed by autolytic enzymes brought in by the tissue fluids which penetrate the area. No cells remain in the treated area that could form bone. If bone should form under these circumstances it would have to be from cells outside the treated area or from cells which migrate into the area by way of the blood stream or by connective tissue ingrowth from the periphery. It would not be expected that any injected osteogenic substance would remain in the area for as long as these experiments ran. Therefore, if bone should develop later, it could hardly be attributed to the injected osteogenic substance. It is also evident that the injury produced by injection of the bone extract, which Heinen[2] believes is responsible for bone formation under these conditions, did not cause bone to develop in the testis. It is true that we did not pretreat the area with 40% alcohol as Annersten[4,5] did, but with the limited area within the testis and the extreme damage to the tissue, it was felt that the effect of alcohol pretreatment would only be to further limit the amount of extract that could be retained. Moreover, Bertelsen[6] has claimed that this pretreatment is not necessary.

The fact that the presence of alcoholic extract of bone and the considerable injury produced by its presence has not caused bone to form in the mouse testis, while marrow transplants readily produce bone there, suggests that in the mouse the primitive mesenchymal cells of the testis are not easily stimulated to form bone. Therefore, the mouse testis is not a good place to test either whether any extractable osteogenic substance exists, as postulated by Annersten[4,5] and Bertelsen,[6] or whether injury to the tissue brought about by the extract is the cause of the response when bone formation follows the injection of alcoholic extracts into muscle.[2] The rabbit, which has been used in the experiments testing the presence or absence of an osteogenic factor, on the other hand is notorious for the ease with which ossification occurs following injury, a fact which has been becoming increasingly evident since the experimental production of ectopic bone in the rabbit by Sacerdotti and Frattin.[18] It seems probable that the present failure to obtain bone formation is related to the use of the mouse as an experimental animal and the testis as the injection site. It was not thought feasible to test the bone extract on the muscle of the mouse since it would be unlikely that appreciably more extract could be injected here than into the testis without affecting the connective tissue involved in tendon or muscle insertion.

Summary. An acid alcoholic extract of bone did not cause the development of bone when it was injected into the testes of 33 male mice of the Strong A strain. It caused extreme damage to about 2/3 of the testis, producing a subtotal infarction of the area. Since fragments of bone marrow produce bone when transplanted to the testis, whereas extracts from much larger amounts of marrow and bone do not cause the development of bone, it is concluded that the marrow reticulum cells survive grafting and produce the bone.

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[18] Sacerdotti, C., and Frattin, G., *Virchows Arch. f. path. Anat.*, 1902, **168**, 431.

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17202. Renal Lesions in Chronic Hypertension Induced by Unilateral Nephrectomy in the Rat.*

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Chronic experimental hypertension induced in the rat resembles that observed in other species with the exception that the incidence of hypertension following operative procedures on one kidney or unilateral nephrectomy is greater than in other species. In the dog, for example, chronic hypertension ensues rarely following application of a clamp to the renal artery, removal of one kidney, or other manipulations, if the opposite kidney be left intact.[1–3] In the rat, on the other hand, approximately 20% of animals develop hypertension following application of a figure-of-eight ligature to one kidney or unilateral nephrectomy.[4] The present study was undertaken to discover the factors which determine whether or not a given animal will develop hypertension following unilateral operation in order to elucidate further the role of the kidney in the pathogenesis of this disorder. The effect of unilateral nephrectomy on the weight of the remaining kidney and the changes produced in this kidney by hypertension; the changes produced in the heart by removal of the remaining kidney; and the influence of various types of diet on the survival period of the bilaterally nephrectomized rat were also studied.

Methods. Healthy piebald rats (Evans-McCollum strain), one to 2 years old, were selected from our breeding colony. The blood pressures were determined daily for a period of 10 days by the plethysmographic method.[5] Under ether anesthesia, the right kidney was removed through a lumbar incision from 110 animals, the blood pressures of which ranged between 110 and 120 mm of mercury. Subsequently, blood pressure determinations were recorded 3 times weekly. The left kidney was then removed and the nephrectomized animals were subjected to various dietary regimes to determine the effects of these diets on the survival period. When the rat died the heart was removed. The weights of each kidney and of the heart were recorded after fixation in 10% formalin. A cross section of the central portion and a longitudinal section of either the distal or the proximal portion of each kidney and a longitudinal or transverse section including both ventricles of the heart were imbedded in paraffin, cut, and stained with hematoxylin and eosin.

Results. Effect on the blood pressure of unilateral nephrectomy. The mean blood pressure levels of 100 rats, 3 months following removal of the right kidney, are recorded in Table I. The figures indicate that 23% of the animals remained normotensive or manifested no greater increase in blood pressure over the preoperative level than the limits of experimental error (± 10 mm of mercury). The largest group, 57%, assumed an intermediate position with blood pressures up to 150 mm of mercury, which are definitely above normal. The remaining 20% of the rats developed hypertension of notable degree with blood pressures above 150 mm of mercury. These are essentially the same effects previously found to follow the application of a figure-of-eight ligature to one kidney, the other kidney being left intact. Following the removal of the second kidney, the blood pressure in those animals which survived to the fifth and sixth days

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*Supported by a grant from the Medical Insurance Fund.

[1] Grollman, A., Factors Regulating Blood Pressure, Trans. Second Conference, Macy Foundation, New York, 1948.

[2] Goldblatt, H., *Physiol. Rev.*, 1947, **27**, 120.

[3] Ogden, E., Tripp, E., and Constant, G., *Fed. Proc.*, 1949, **8**, 120.

[4] Grollman, A., Harrison, T. R., and Williams, J. R., Jr., *Am. J. Physiol.*, 1943, **139**, 293.

[5] Williams, J. R., Jr., Harrison, T. R., and Grollman, A., *J. Clin. Invest.*, 1939, **18**, 373.

TABLE I.
Incidence of Lesions in the Kidney and Blood Pressure of 100 Rats Following Unilateral Nephrectomy.

Mean blood pressure in mm Hg	Up to 120	120 to 130	130 to 140	140 to 150	Over 150
No. of rats	8	15	29	28	20
Incidence of lesions in ablated kidney	0	+	++	+++	++++

TABLE II.
Effect of Various Diets on the Survival of Rats Following Bilateral Nephrectomy.

No. of rats	Diet	Survival in days	
		Range	Avg
20	Regular	2.0 to 4.5	3.0 ± 0.4*
20	Dialyzed (sodium and potassium "free")	3.2 to 6.0	4.5 ± 0.4
20	"Protein-free" diet (glucose, starch, lard)	2.9 to 5.5	4.6 ± 0.5
20	Dialyzed food plus 2% NaCl	2.8 to 5.0	4.3 ± 0.5
20	" " " " KCl	2.5 to 4.5	3.7 ± 0.3

* Standard deviation.

was in each case found to be elevated 30 to 50 mm of mercury over the highest level attained prior to the removal of the second kidney. Bilateral nephrectomy in the rat, as in the dog,[6] thus results in elevation of the blood pressure to hypertensive levels if the animals survive for 5 days or more in good condition.

Microscopic studies of the right kidneys. The structural deviations from normal observed microscopically in the right kidneys of the rats whose blood pressure levels are recorded in Table I could be grouped as absent, minimal, and slight. There was a conspicuous absence of any acute or chronic inflammatory change involving the glomeruli, tubules, renal pelvis, stroma or blood vessels. The minimal and slight changes observed consisted of an increase in the connective tissue stroma of occasional glomeruli and hyaline change of some glomeruli, of varying degrees or complete. Glomeruli with such changes were few and only in occasional kidneys were they associated with changes in the tubules. Comparison of the incidence of these structural changes in the kidneys with the blood pressures of the animals from which they were removed indicated with some degree of certainty that glomeruli with hyaline change were more numerous and that the associated tubular changes were conspicuous in those rats whose blood pressures were in the hypertensive range. The structural changes were absent or minimal in the kidneys of animals, the blood pressures of which were not affected by nephrectomy.

Survival following removal of the second kidney. The survival times of the rats following removal of their second kidney are recorded in Table II. Removal by dialysis of the salts present in the regular diet or the use of a salt-free, protein-free diet increased the average survival times from 3 to 4.5 days. That this prolongation in the survival time is due chiefly to the removal of potassium salts from the diet is shown by the fact that the addition of sodium chloride to the dialyzed diet did not affect appreciably the period of survival, whereas the addition of potassium chloride reduced it to a period comparable to that observed with the regular diet. Complete abstinence from food or the administration of 0.9% saline in lieu of drinking water also reduced the survival period markedly. The longest survival observed under optimal conditions was 6 days. Comparable results have been reported for the dog.[6] The urea content of the blood of 18 of the bilaterally nephrectomized rats just prior to or at death varied from 500 to 1090 mg per 100 cc and averaged 748 mg %. These values are appreciably higher than those reported by

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[6] Grollman, A., Vanatta, J., and Muirhead, E. E., *Am. J. Physiol.*, 1949, **157**, 21.

TABLE III.
Relation of Weight of Kidneys and Heart to Ultimate Blood Pressure.

Ultimate mean blood pressure in mm Hg	Up to 120	120 to 130	130 to 140	140 to 150	Over 150
	Avg wt in g per 100 g of body wt				
Right kidney	0.46	0.46	0.48	0.50	0.49
Left ”	0.46	0.48	0.50	0.53	0.55
Heart	0.31	0.33	0.34	0.36	0.48

others,[7-8] and reflect the much longer survival period of our animals which are comparable to those reported by Rosenkrantz,[9] who administered aluminum hydroxide to prolong the life of his rats. The longer survival of our animals on a regular diet, we attribute to the fact that removal of the second kidney was accomplished with a minimum of trauma.

Studies on the remaining kidney and heart. The average weight in grams of each kidney and heart calculated per 100 g of body weight are recorded in Table III. The weights of the right kidneys were essentially the same in all animals regardless of the ultimate blood pressure attained following unilateral nephrectomy. The weights of the left kidneys, on the other hand, were increased in proportion with the elevation of blood pressure. It will be noted that removal of kidneys which on microscopic examination were normal resulted in no increase in weight of the remaining kidney whereas the kidneys of animals manifesting microscopic changes underwent maximum enlargement. There was thus no correlation between the weight of the first kidney removed and the ultimate blood pressure but a definite correlation between that of the remaining kidney and the blood pressure. The weights of the hearts increased proportionately to the elevation of blood pressure. This hypertrophy is not reflected exactly by the data of Table III since some loss of substance was likely to occur during the survival following removal of the second kidney. Microscopic studies of the left kidneys disclosed no striking differences from the right kidneys of the same rats. Occasional glomeruli seemed larger and the tubules perhaps more spacious. This might account for the slight increase in weight of the kidneys. Changes also occurred in the left kidneys of those rats in which lesion of the right kidneys were minimal or slight. In these, glomeruli with hyaline change were more numerous and the associated tubules were dilated by a homogeneous, bright pink material and were lined by flat cells. These changes were marked only in those rats which developed hypertension. Typical appearances of the kidneys are shown in Fig. 1. Microscopic studies of the hearts disclosed minimal or slight hypertrophy of the left ventricle. The increase and variation in size of the myocardial fibers were most marked in the hypertensive rats. Acute changes were superimposed in all of the hearts which presumably occurred during the period following removal of the second kidney. They consisted of varying degrees of dilatation of the capillaries with focal areas of extravasation of blood and obliteration of the striations of the myocardial fibers to a degree of waxy change. These changes are comparable to those observed in the nephrectomized dog.[10] No acute or chronic inflammatory lesions were noted in the stroma nor were there any obvious changes in the blood vessels.

Discussion. The results of the present study are compatible with the view that the maintenance of a normal blood pressure is dependent on the presence in the organism of an adequate renal function.[11] It is impossible, on the other hand, to harmonize the present observations with the view that hy-

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[7] Durlacher, S. H., and Darrow, D. C., *Am. J. Physiol.*, 1942, **136**, 577.

[8] Bondy, P. K., and Engel, F. L., PROC. SOC. EXP. BIOL. AND MED., 1947, **66**, 104.

[9] Rosenkrantz, J. A., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 155.

[10] Muirhead, E. E., Vanatta, J., and Grollman, A., *Arch. Path.*, in press.

[11] Grollman, A., *Special Publications N. Y. Acad. Sci.*, 1946, **3**, 99; Factors Regulating Blood Pressure, First Conference, Macy Foundation, New York, 1947.

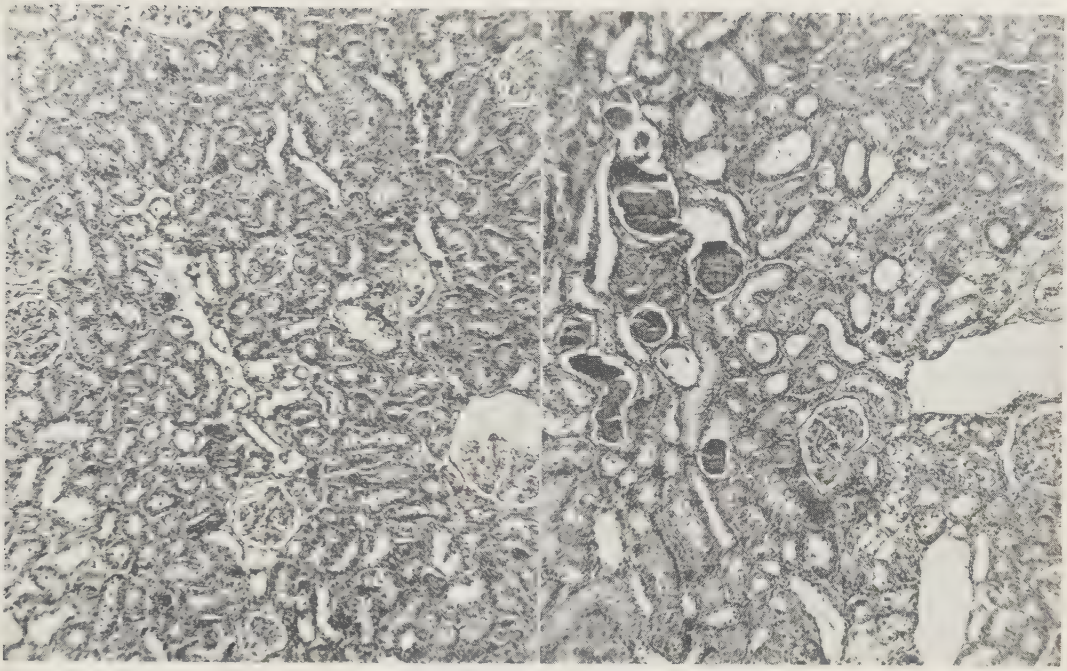

FIG. 1.
Microscopic changes in glomeruli and tubules of the right kidney (left) removal of which was followed by permanent elevation of blood pressure to 160 mm Hg. More extensive changes are seen in the left kidney (right) of the same rat (R-64-48) removed 10 weeks later. × 60.

pertension results from the elaboration of some pressor agent unless one makes the unlikely assumption that removal of one kidney puts such a strain on the other that it excretes a pressor agent.[12] When both kidneys are unimpaired and one kidney is removed, the other may compensate for the removed kidney and no hypertension results. On the other hand, when both kidneys are slightly impaired and one kidney is removed, an elevation in blood pressure ensues. The fact that unilateral nephrectomy in the rat more frequently results in hypertension than the same procedure does in the rabbit[13] or in the dog[14] suggests that the rat has less reserve of kidney function than do these other experimental animals. Microscopic examination of presumably normal kidneys supports this hypothesis. However, the lesions present in the rat are not, as commonly stated, a result of preexistent pyelonephritis. They resemble (Fig. 1) those described by the authors[15] in rats with chronic hypertension, but are much less extensive. As regards the nature of the lesions, they apparently are not vascular in origin[16] but are probably secondary to nutritional deficiencies in early life. Such deficiencies induce renal damage[17] which, if extensive, results in hypertension.[18] This would account for the observation that the incidence of spontaneously occurring hypertension in rats varies and depends on the dietary conditions under which they have been reared. A group of rats, for example, which as weanlings had been used in dietary deprivation studies, were found to have an incidence of about 20% of hypertension, whereas those maintained on adequate diets rarely developed the disorder spontaneously. The existence of lesions comparable to those observed would account for the rises in blood pressure observed in the rabbit and dog following unilateral nephrectomy.[6,13,14]

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[12] Goldblatt, H., The Renal Origin of Hypertension, Chas. C. Thomas, Springfield, 1948.

[13] Grollman, A., *Am. J. Physiol.*, 1942, **142**, 666.

[14] Grollman, A., *Am. J. Physiol.*, 1946, **147**, 647.

[15] Halpert, B., and Grollman, A., *Arch. Path.*, 1947, **43**, 559.

[16] Halpert, B., and Grollman, A., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 273.

[17] Heller, H., and Dicker, S. E., *Proc. Roy. Soc. Med.*, 1947, **40**, 351; Dessau, F. I., and Oleson, J. J., *Proc. Roy. Soc. Exp. Biol. and Med.*, 1947, **64**, 278.

[18] Hartcroft, W. S., and Best, C. H., *Brit. Med. J.*, 1949, **1**, 423.

Summary. The incidence of hypertension following unilateral nephrectomy is recorded in 100 rats. The development of hypertension was shown to be dependent on the status of the kidneys at the time of removal of the first kidney. If lesions are present, hypertension develops; if absent or minimal, the animals remain normotensive. The bearing of these observations on current theories as to the pathogenesis of experimental hypertension are discussed.

The effects of unilateral nephrectomy on the weight of the heart and remaining kidney and the influence of various types of diet on the survival period following removal of the remaining kidney were also determined. Enlargement of the heart as a result of hypertrophy, as well as of the remaining kidney, may be correlated with the degree of hypertension induced by unilateral nephrectomy. Removal of potassium from the diet markedly prolongs the survival period following bilateral nephrectomy.

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17203. Limiting Essential Amino Acids of Some Legume Seeds.

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Pulses are one of the most important sources of vegetable proteins in human diets and knowledge of their amino acid composition is of interest especially in countries where there is insufficient consumption of animal products. The present experiments are part of a study on the nutritional problem in Venezuela, where legumes play a very important role in human nutrition.

The experiments were performed with young rats of the Sprague-Dawley strain. Each group consisted of 2 male and 2 female animals, housed individually in screen-bottomed cages. In most cases the animals were put on the experimental diet at the age of 3 weeks and weighed about 40 g each; in some cases animals weighing about 100 g were used. The diets used were similar to those described by Russell *et al.*[1] and contained 10% of protein and 4-5% of fat, with the exception of the soybean diets which contained 7% of fat. Food and water were given *ad libitum*. All animals received 3 drops of liver extract 3 times weekly by dropper; this amount was sufficient to promote maximum growth on a soybean-corn diet when tested by a method previously described.[2] Each group received the unsupplemented diet for 3 weeks, immediately followed by the supplemented diet for the same time. In some cases the diets were given for 10 periods only. Animals were weighed every 5 days and food consumption was recorded. Digestibility was determined for all the legumes studied. Since good correlation between growth and protein efficiency was observed in accordance with the results of other authors,[3] only weight changes are reported.

The legume seeds were obtained from the Genetical Division of the Ministry of Agriculture in Maracay or were purchased from the local market. All seeds were ground and autoclaved at 10 lb. pressure for 20 minutes with the exception of the kidney and hyacinth beans, which were soaked in water over night, autoclaved for 30 minutes at 10 lb. pressure,

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[1] Russell, W. C., Taylor, M. W., Mehrhof, T. G., and Hirsch, R. R., *J. Nutr.*, 1946, **34**, 491.

[2] Jaffé, W. G., and Elvehjem, C. A., *J. Biol. Chem.*, 1947, **169**, 287.

[3] Hegsted, D. M., and Worcester, J., *J. Nutr.*, 1947, **33**, 685.

TABLE I.
Weight Changes of Rats Receiving Diets Containing Legumes as Only Source of Protein, With, or Without, Methionine Supplementation.

Legume	Scientific name	No. of samples studied	Wt change/ day/animal on basal diet	Wt change/ day/animal on diet supplemented with 0.3% methionine
Black kidney beans	*Phaseolus vulgaris*	4	0.0	3.1
Red " "	" "	3	—0.1	2.5
White " "	" "	1	0.9	2.6
Adzuki "	" *angularis*	1	1.0	1.8
Mung "	" *aureus*	1	0.5	2.3
Cow peas	*Vigna sinensis*	4	0.3	1.5
Soy beans	*Glicina soja*	3	1.3	2.5
Split peas	*Pisum sativum*	2	—0.1	0.9
Dried green peas	" "	1	0.2	1.7
Lentils	*Lens esculenta*	2	—0.1	0.5
Chick peas	*Cicer arietinum*	2	1.3	2.9
Hyacinth beans	*Dolichos lablab*	2	—0.3	4.4
Pigeon peas	*Cajanus indicus*	4	0.2	0.3*
Casein		1	2.0	4.2

* Supplemented with 0.075% tryptophan 0.2
" " " " and 0.3% methionine 3.1

then dried and ground. This procedure was adapted because these seeds contained a toxic principle in the raw stage which has been found not to disappear completely after autoclaving the dry ground material.[4]

With the exception of soybeans and chick peas, none of the legumes studied promoted more than very slight growth in rats if present in the diet in amounts corresponding to 10% of protein. Dried split peas, lentils and pigeon peas did not cause good growth even when fed together with methionine. Diets containing kidney beans or hyacinth beans supplemented with methionine promoted as good or better growth than did soybeans supplemented with methionine and comparable with casein.

The data show that under the experimental conditions, methionine was a limiting indispensable amino acid in all the samples. In the cases where previous studies exist, our results are in accordance with published data.[1,5-7] The results obtained with pigeon peas show that the value of the proteins for growing rats is limited by both methionine and tryptophan, as supplementation with one of these alone caused no growth response, while a combination of both resulted in enhanced weight increase.

Summary. The essential amino acid of legume seeds limiting growth of young rats was methionine in all cases studied, with the exception of pigeon peas, in which methionine and tryptophan were both limiting under the experimental conditions.

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[4] Jaffé, W. G., *Experientia*, 1949, **5**, 81.

[5] Block, R. J., and Michell, H. H., *Nutr. Abstr. and Rev.*, 1946, **16**, 249.

[6] Richardson, L. R., *J. Nutr.*, 1948, **36**, 451.

[7] Belton, W. E., and Hoover, C. A., *J. Biol. Chem.*, 1948, **175**, 377.

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17204. Construction and Application of Shielded Silver Electrodes with Mercury Leads Designed for Prolonged Stimulation Experiments.*

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Prolonged electrical stimulation of the nervous system of conscious animals requires shielded electrodes which will not injure the nerves or cause undue local tissue reaction. The leads connecting the electrodes to the external electrical circuit must be capable of withstanding the almost continuous motion of a normal animal for periods of several months. The purpose of this report is to describe in detail the construction of electrodes and leads which have fulfilled the above requirements. The production of arterial hypertension by renal artery-nerve or splanchnic nerve stimulation with similar electrodes has been reported.[1-4]

The electrodes are constructed of No. 18 fine silver wire and are imbedded in a molded lucite block (Fig. 1). The silver electrode wire is exposed on the inner surface of the lucite block to provide direct contact with the nerve. The obturator occludes the slot in the block after the nerve is in place. The hollow stainless steel terminal is filled with mercury and connected to a mercury-filled, double lumen rubber tube which provides electrical contact to the external electrical circuits.

A double lumen latex tube joined in a Y at the stainless steel terminal provides a means of flushing and cleaning the mercury columns whenever necessary. 1N HCl, followed by water, alcohol and air, is injected into one tube, flowing through that tube, through the hole in the stainless steel terminal and out through the second tube. Then this system is refilled with clean mercury.

Construction of electrodes. The electrode blocks are molded from powdered lucite† at a temperature of 175°C and a pressure of approximately 1000 lbs./in.^{2} (Fig. 2).

Step 1: (Fig. 2, photo 1). The silver wire (b) is wrapped around the mandrel (c) and soldered to the stainless steel terminal (a). Soldering flux designed for stainless steel facilitates this step.‡

Step 2: The plate (h) (Fig. 2, photo 4) is placed in the bottom of the holder (j). The assembly in (1) is then placed in the holder (j) with the flat portion of the mandrel (c) inserted into the slot in the plate (h). The screw (k) is then tightened. The guide (g) (Fig. 2, photo 5) is now placed in the holder (j) and the cylinder (d) (photo 2) is attached to the holder (j). The entire assembly is filled with powdered lucite to about ⅛ inch from the top of the cylinder. Tapping the assembly will insure complete filling. The piston (e) (photo 2) is inserted into the cylinder and the complete unit is placed in the press. The springs are tightened until they are nearly closed.§ The press and molding assembly is placed in an oven at 175°C. After 20 minutes the springs are again tightened and the entire apparatus is returned to the oven for 15 minutes. After the final baking the mold is allowed to cool slowly to room temperature before releasing the spring tension. When the cylinder (d) (Fig. 2,

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* Supported by a grant from the Life Insurance Medical Research Fund.

[1] Kottke, F. J., Kubicek, W. G., and Visscher, M. B., *Am. J. Physiol.*, 1945, **145**, 38.

[2] Kubicek, W. G., and Kottke, F. J., *Fed. Proc.*, 1946, **5**, No. 1.

[3] Kottke, F. J., and Kubicek, W. G., *Fed. Proc.*, 1947, **6**, No. 1.

[4] Kubicek, W. G., and Kottke, F. J., *Fed. Proc.*, 1947, **6**, No. 1.

† E. I. DuPont de Nemours Co., Plastic Dept., Arlington, N. J. Crystal, Color H7500, Comp. HG 22, Gran Fine No. H18876.

‡ The Lloyd S. Johnson Co., 2241 Indiana Ave., Chicago, Ill. Stainless steel soldering flux.

§ Eiler Spring Co., 637 Sexton Building, Minneapolis, Minn. .125″ wire coil compression springs, 10 turns 2 inches in length, ½″ inside diameter, approximately 90 lbs. to compress spring completely.

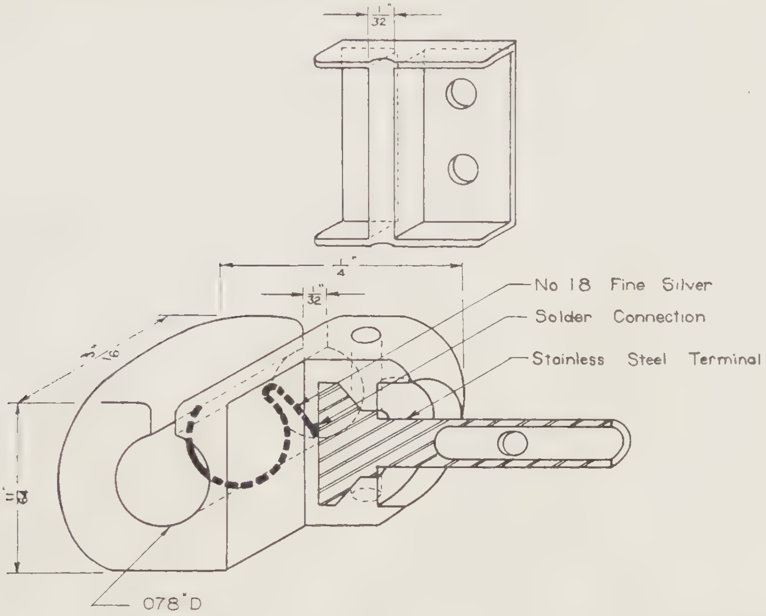

FIG. 1.
Pictorial diagram of lucite electrode block and obturator.

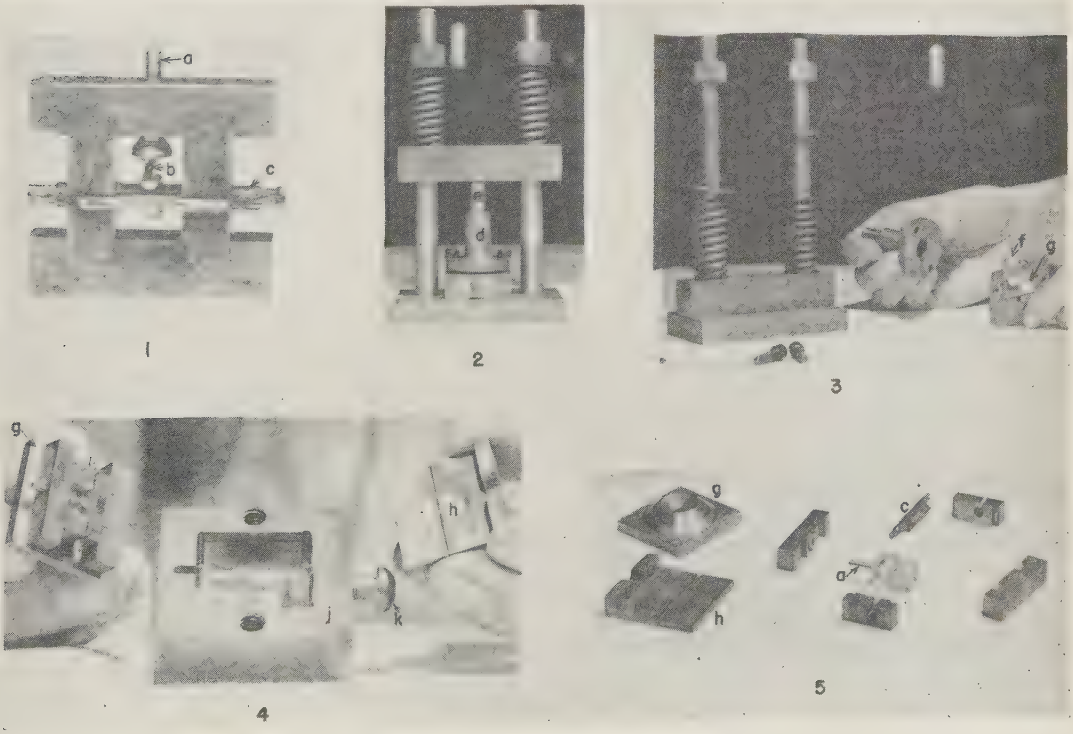

FIG. 2.
A composite photograph of the various steps in the molding process.

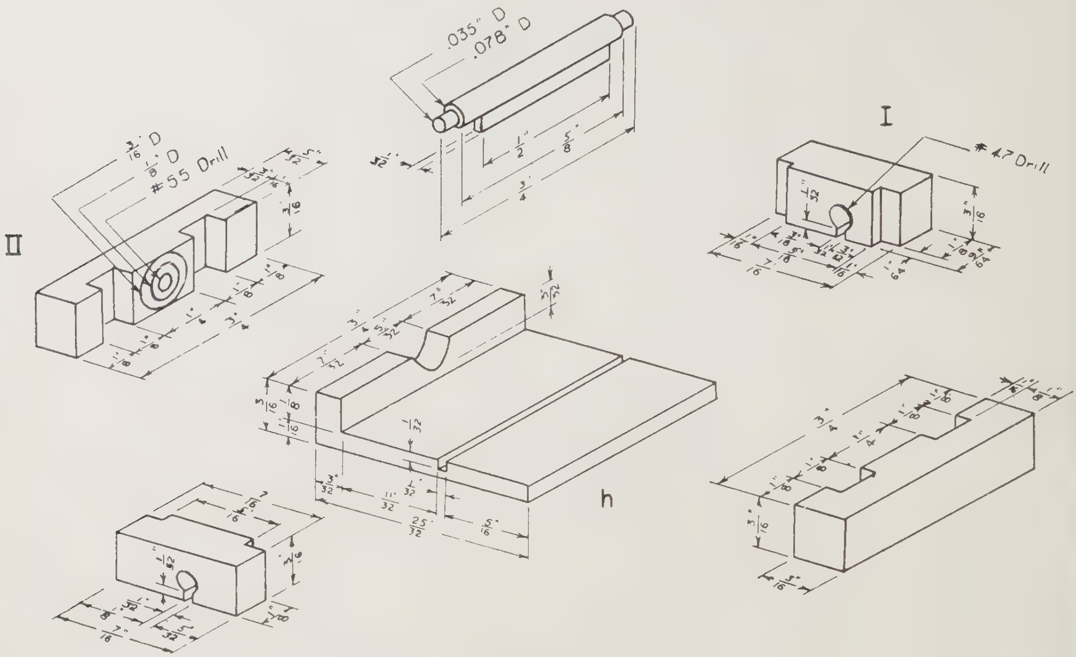

FIG. 3.
Assembly drawing of electrode mold.

photo 3) is removed, a lucite bar (f) is found attached to the bottom of the electrode block. This bar is twisted off and the guide (g) removed. The unit in photo 1 is placed astraddle a hole in a piece of wood in such a way as to allow the mandrel (c) to be driven out with a punch.

Step 3: Obturators (Fig. 1) are molded from powdered lucite by forcing molten lucite into a plaster cast of a wax impression of the slot in the electrode block. To form a wax impression, the electrode block is first painted with an aqueous soap solution. Inlay casting wax is softened over a flame and forced into the slot with pressure applied with the thumb. Molten wax is then added to strengthen the impression. The excess wax is trimmed off leaving small tabs at the corners to act as guides on the obturator (Fig. 1). After cooling under tap water a No. 48 drill is passed through the electrode block to remove the excess wax protruding into the lumen of the block and to shape the obturator in such a way as to fill completely the slot in the block and to leave the interior of the block smooth and cylindrical when the obturator is in place.

The plaster cast is made in a small metal box with mortised joints similar to the mold (Fig. 3). The box is .5″ deep, .4″ wide, and .75″ long inside with ⅛″ walls. A holder similar to (j) (Fig. 2) is used to support the cylinder (d) and the casting box. The guide (g) is not necessary in molding the obturators. When the box is filled with fine thoroughly mixed plaster the surface of the wax impression is painted with plaster to insure close adherence to the contour of the impression. The wax is then immersed, impression down, in the plaster. After setting, the excess plaster is chipped off and the wax removed after heating under hot tap water. Before filling the cast with lucite the surface of the plaster is painted with a sealing agent.[‖] The filling and baking procedure is the same as described for the electrodes. The obturator is formed on the end of a lucite bar (f, Fig. 2, photo 3) and is sawed off after the plaster has been chipped off. (A weak solution of HCl is useful in removing the last bits of plaster). The obturator is then slipped into place on the electrode block. After

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[‖] L. D. Caulk Company, Milford, Dela. Al-Cote sealing agent.

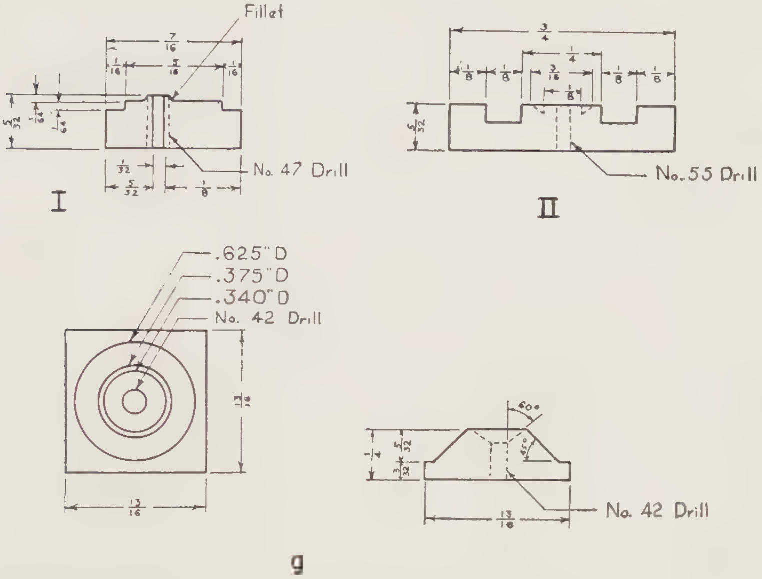

FIG. 4.
Detail drawing of mold parts I and II of Fig. 3 and guide (g) (Fig. 2) (photo 5).

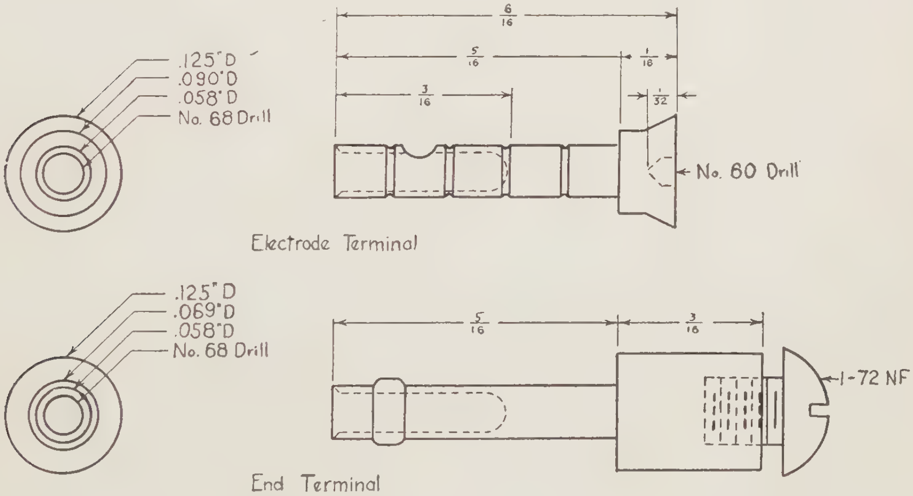

FIG. 5.
Detail drawing of electrode terminal and end terminal.

rounding off the corners and sharp edges the lucite is polished and the holes drilled for the ligatures (Fig. 1). Lubricating the drill with kerosene will facilitate drilling lucite. During the molding process a thin film of lucite forms over the silver electrode. The film is removed with a dental grinder.

Details of the molding apparatus are presented in Fig. 3-7.

Step 4: *Construction of small double lumen latex tubes.* Stainless steel wires are joined with Wood's metal (Fig. 8). The wires are then dipped into a clay or mica release medium,¶ then into a coagulant,¶ and finally into latex.¶ The wall thickness of the

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¶ American Anode Company, Akron, Ohio. Clay or mica release medium, No. 545 coagulant 60% centrifuged latex (keep latex at pH 9).

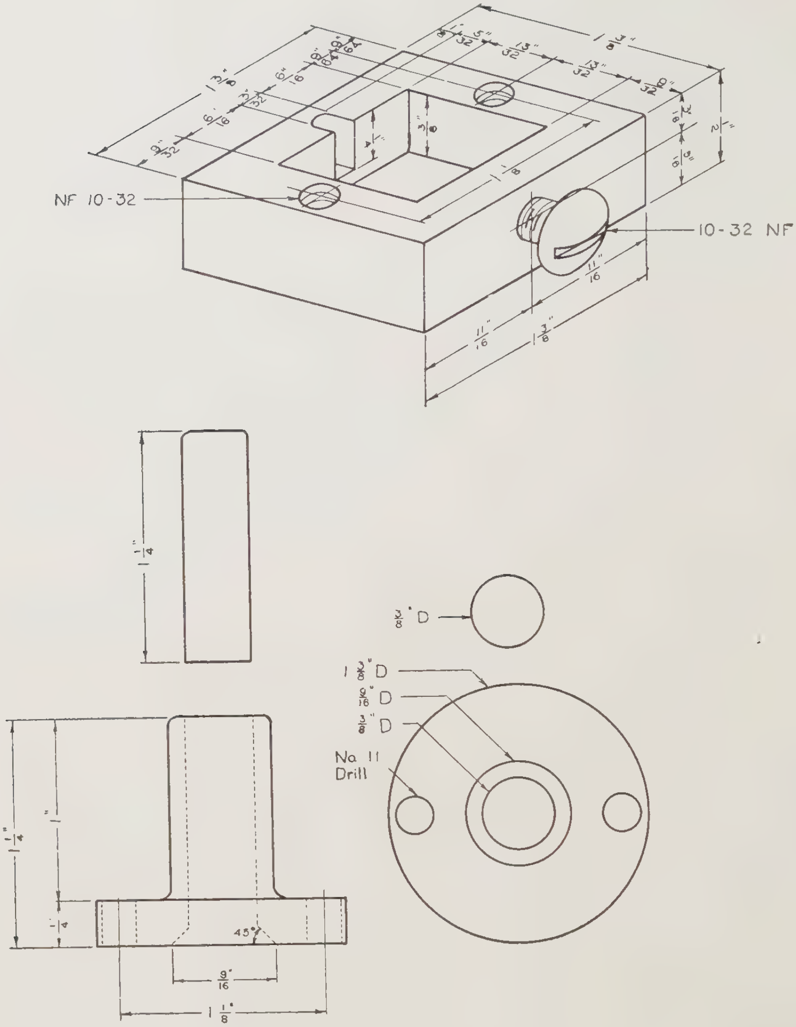

Fig. 6.
Pictorial drawing of holder (j) and detail of cylinder (d) and piston (c) (Fig. 2).

tubes is controlled by the length of time allowed for deposition of latex on the wires. Usually 5 to 10 minutes is sufficient. The wires are carefully removed from the latex and approximately one hour allowed for coagulation to proceed. The wires and tubes are then washed in tap water for one hour, rinsed in distilled water, and dried until all white color has disappeared. Twenty-four hours at room temperature or 12 hours at 50°C will usually complete the drying. The tubes are finally cured for 20 minutes at 105°C. The tubes are removed quickly from the curing oven and the wires separated at the Wood's metal junction. Later the wires are removed, the excess rubber is trimmed off, and the tubes cleaned with 1N HCl, water, alcohol, and dried with air. The tube extending past the junction of the lumina should be cut as short as possible to insure continuity of the junction of the tubes and the hole in the stainless steel terminal in the electrode block (Fig. 1).

Step 5: *Assembling the electrode and leads.* The hollow portion of the electrode terminal is filled with mercury with a syringe and needle. The terminal is inserted into the

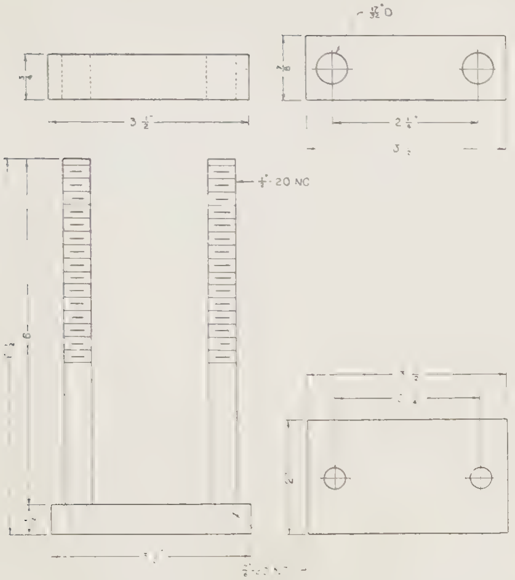

FIG. 7.
Detail drawing of press, (Fig. 2) (photo 2).

straight rubber tube until the hole in the side of the terminal coincides with the junction of the lumina. Pushing the terminal beyond this point and withdrawing to the proper position will produce a more reliable connection. Two or 3 turns of thread below the junction aids in securing the rubber to the terminal. Water-proof cement between the rubber tube and the lucite block insures good electrical insulation of the terminal. The hollow stainless steel terminal for the distal end of the tube is filled with mercury (Fig. 5). With a smooth 18-gauge needle and syringe 5 cc of mercury are flushed rapidly through the rubber tube and electrode terminal. Without allowing air to enter either tube and with the syringe in place the mercury in the distal terminal is joined by cohesion to the mercury in one lumen of the rubber tube and the terminal inserted. To insure complete filling the needle is re-

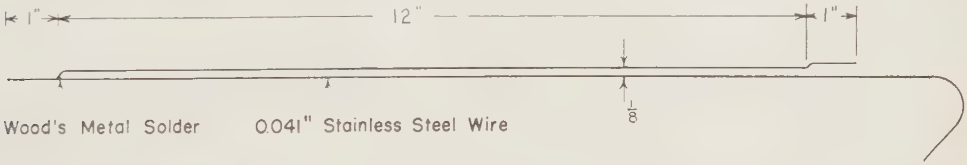

FIG. 8.
Diagram of stainless steel wires used for constructing double lumen latex leads.

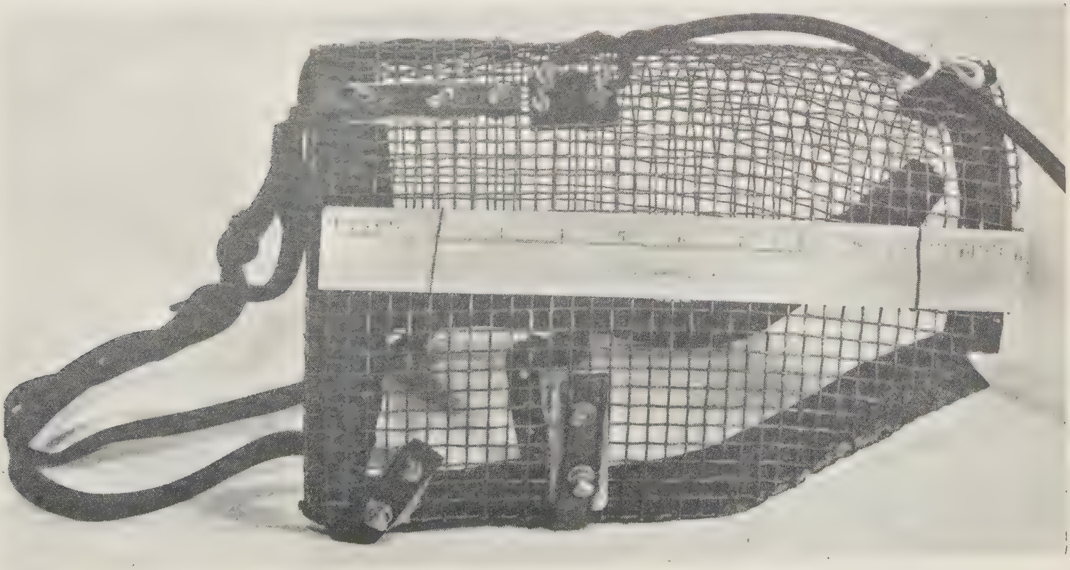

FIG. 9.
Photograph illustrating the gravel screen saddle, electrode terminal block, and lamp cord connecting the electrodes to the stimulator.

moved with the rubber tube in the vertical position since the rubber will distend slightly due to the pressure of the mercury column. When the remaining rubber tube is completely filled with mercury a metal obturator is inserted and several turns of thread applied tightly around both the obturator and the distal terminal.

Application of electrodes. The splanchnic nerves are exposed through bilateral dorsal incisions. The nerves are carefully dissected free of surrounding tissue. An electrode is placed around each nerve and the obturator tied in place. Two or three turns of linen ligature around the rubber tube and through adjacent fascia holds the electrode in position so that the course of the nerve is altered as little as possible. The mercury lead is then brought through the incision by a circuitous route and finally through the skin approximately two inches from the spine at about the level of the last rib. Anchoring the leads to bony structures and fascia at several points aids in securing the electrode in the proper position and prevents any pull directly on the nerve. Small flexible wire leads connect the mercury leads to a terminal block on the gravel screen saddle used to protect the leads (Fig. 9). The animal is tied to one end of a rectangular cage which allows him to lie down or stand at will but prevents turning completely around, thus preventing destruction of the leads to the stimulator.

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17205. Urinary Excretion of Digitoxin in the Rat.*

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Heretofore it has not been possible to measure the actual renal excretion of digitalis glycosides. However, by means of the embryonic duck heart method, we have been able to measure in a quantitative fashion minute amounts of digitoxin (Sandoz) in the 24-hour urine volumes of the rat.

Methods. The 24-hour urine sample of a rat is concentrated to 10 ml volume by boiling. Three grams of infusorial earth are added and the mixture dried at 80° *in vacuo.* The dried residue is broken up with a glass rod, 50 ml of U.S.P. grade chloroform is added, and the mixture is shaken for one hour in a mechanical shaker. The chloroform extract is separated by filtration through paper. The residue on the paper is washed 3 times with fresh 10 ml portions of chloroform, the solvent being pressed out of the residue after each washing. The washings are combined with the filtrate, the extract is concentrated to a small volume by boiling, and is then dried at 80°C *in vacuo.* The dried extract is taken up in 1 ml of ethanol by shaking for one hour. Ten ml of water is added to the alcohol and the solution is concentrated to 1 ml by boiling. Another 10 ml of water is added and the solution again concentrated to 1 ml. This concentrated solution is diluted to a final volume of 60 ml by the addition of 59 ml of Tyrode's solution (without glucose). Embryonic duck hearts are exposed to this latter solution and the time of "digitalis effect"[1] observed and the quantity of digitoxin estimated according to previously described methods.[2] It thus was found possible to detect as well as measure as little as one microgram of digitoxin added to the 24-hour urine sample of the rat. A quantitative differentia-

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* Aided by grants from The Life Insurance Medical Research Fund, The U. S. Public Health Service, and The Sandoz Chemical Works.

[1] Friedman, M., and Bine, R., Jr., Proc. Soc. Exp. Biol. and Med., 1947, **64**, 162.

[2] Bine, R., Jr., and Friedman, M., *Am. J. Med. Sci.*, 1948, **216**, 534.

tion could be made between urine samples containing different quantities of added digitoxin.

Ten albino rats (average weight 165 g) were given 100 μg of digitoxin per kilogram of body weight by intravenous injection, and their urine was collected for 24 hours. The urine samples of 5 of these rats were also collected on the second and third day following injection. Twelve rats (average weight 223 g) were given 1000 μg of digitoxin per kilogram of body weight and similar urine collections were made during the first 24 hours. Urine collections of eight of these rats also were made on the second and third days following injection. All urine samples were extracted and tested on the duck hearts as described above.

Results. The injection of a moderately large amount of digitoxin (*i.e.*, 100 μg/kilo) into 10 rats was not followed by the detectable appearance of digitoxin in the 24-hour samples of urine of any of the rats either 24, 48, or 72 hours after the injection. In view of the fact that each rat actually received approximately 16.5 μg of digitoxin, absence of as much as one microgram of digitoxin in the urine indicated that little or no digitoxin was excreted by the kidneys of these rats.

The urine samples, however, collected during the first 24 hours in the 12 rats who had received 10 times the above amount of digitoxin (1000 μg/kilo) contained an average of 6 μg digitoxin (Range, 4 to 10 μg). However, none of the urine samples collected during the second or third days after injection contained a detectable amount of digitoxin (*i.e.*, less than 1 μg).

Thus, even in these rats which had received a relatively tremendous amount of digitoxin (average, 223 μg) the average renal excretion of only 6 μg (less than 3% of the administered dose) again indicates that the renal excretion of digitoxin in the rat is negligible. These results, however, are not to be construed as evidence for the negligible excretion of other cardiac glycosides in the rat. Moreover, these results may not be valid for other species in view of the fact that the rat appears to react anomalously to the administration of digitoxin.

Conclusion. A method for the quantitative determination of minute amounts of digitoxin in urine is described. The renal excretion of digitoxin is negligible in the rat.

The authors wish to express their appreciation to Vivian Seay and Fred Michaelis for their technical assistance.

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17206. Gonadotrophic Activity of Granules Isolated from Rat Pituitary Glands.*

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The respiratory enzyme systems, cytochrome oxidase and succinoxidase, have been shown to be associated to a large extent with a large granule or mitochondrial fraction isolated by differential centrifugation of alkaline water homogenates (Schneider[1]), and saline extracts (Hogeboom, Claude and Hotchkiss[2]) of rat liver. Subsequently morphologically intact mitochondria were isolated by differential centrifugation of 0.88 M sucrose homogenates of rat liver and kidney (Hogeboom, Schneider and Pallade[3,4]). On

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* Supported in part by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.

[1] Schneider, W. C., *J. Biol. Chem.*, 1946, **165**, 585.

[2] Hogeboom, G. H., Claude, A., and Hotchkiss, R. D., *J. Biol. Chem.*, 1946, **165**, 615.

[3] Hogeboom, G. H., Schneider, W. C., and Pallade, G. E., Proc. Soc. Exp. Biol. and Med., 1947, **65**, 320.

[4] Hogeboom, G. H., Schneider, W. C., and Pallade, G. E., *J. Biol. Chem.*, 1948, **172**, 619.

the basis of these results it seemed that it might be possible to isolate granule fractions from fresh pituitary tissue, and study them not only from the standpoint of their enzyme activity but also from the standpoint of whether gonadotrophic hormone is associated with them. Results of preliminary experiments using rat pituitary gland are given in this report.

Experimental. The pituitary glands were obtained from 4 groups of 50 to 60 normal adult rats (Holtzman strain) and one group of 35 adult rats castrated for 3 months. The animals were killed by decapitation. The glands were removed immediately, dissected free of the pars nervosa, and the adenohypophyses were placed on moist hard filter paper in a Stender dish which was kept on ice until the collection was completed. The glands were weighed and placed in a sharp pointed homogenizing tube that contained 0.4 ml of cold 0.88 M sucrose. The tube was kept in an ice bath at all times except during homogenization. The glands were finely homogenized in 19 volumes of 0.88 M sucrose which was the concentration of sucrose used by Hogeboom, Schneider and Pallade.[3,4]

The pituitary homogenates were fractionated by differential centrifugation in the multispeed head of an International refrigerated centrifuge kept at 2°C. The method of fractionation as to time and speed of centrifugation is similar to that reported by Schneider[5] for the isolation of mitochondria from isotonic sucrose (8.5 g sucrose per 100 ml) homogenates of rat liver. The liver mitochondria were washed with isotonic sucrose and they were shown to contain the major portion of the succinoxidase activity of the homogenates. It should be pointed out that the granule fractions isolated from the pituitary homogenates were washed with 0.9% NaCl and this resulted in the separation of the gonadotrophin from these fractions. The method of fractionation is as follows:

1. The whole homogenate designated as *A* was centrifuged at approximately 500 × g for 10 minutes. The opaque extract was removed and the residue was washed twice by suspending in 0.88 M sucrose and centrifuging in the same way. The washings were combined with the above sucrose extract. The residue which consisted of cell nuclei and other insoluble material was designated as *B*.

2. The extract was centrifuged at 8,000 × g for 20 minutes. The aqueous sucrose extract was removed. The recovered precipitate (large granule fraction) was designated as *C*, and it was washed once by suspending in 0.88 M sucrose and centrifuging. The washings were added to the sucrose extract. The large granule fraction was suspended in 0.9% NaCl and centrifuged. This treatment was repeated one time. The washed fraction was suspended in NaCl and designated as *D*, and the combined NaCl washings as *E*.

3. Fractions containing small granules were recovered from the aqueous sucrose supernatant fractions of 2 groups of normal and the group of castrate rats by centrifuging for one hour at 15,000 × g. The small granule fraction was designated as *F*; it was washed twice with 0.9% NaCl. The washed granules were designated as *G*, and the washings as *H*. The aqueous sucrose extract from which the granules were removed was designated as *I*.

The fractions obtained by the above procedure were stained by a modification of the acid fuchsin methyl green method of Bensley[6] as an aid in estimating the homogeneity of the fractions.

The above fractions were assayed for gonadotrophic activity by use of 21-day-old female rats of the Holtzman strain. The volumes of the fractions were adjusted with saline for purposes of injection. Each rat received 0.5 ml of the proper fraction on the afternoon of the first day, and 0.5 ml on the morning and afternoon of the next 4 days. Autopsy was performed on the morning after the last injection. The ovaries were removed, dissected free of other tissues and weighed. The qualitative effect as to the presence of follicles and corpora lutea was recorded. The degree of stimulation of the uteri was also noted.

The succinoxidase activity of the whole sucrose homogenate and the large granule fraction was determined by the use of a con-

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[5] Schneider, W. C., *J. Biol. Chem.*, 1948, **176**, 259.

[6] Bensley, R. R., *Am. J. Anat.*, 1911, **12**, 297.

TABLE I.
Gonadotrophic Activity of Fractions Obtained from Fresh Rat Pituitary Glands by High Speed Centrifugation.

Kind of fraction	Total amount of fraction injected, mg*	Normal rats (4 groups) Avg wt ovaries, mg	Castrate rats (1 group) Avg wt ovaries, mg
A. Whole homogenate	5		89 (3)†
	10	126 (5)†	153 (3)
	20	190 (9)	
B. Residue (nuclei and connective tissue)	20	15 (10)	18 (3)
	100	24 (2)	47 (2)
C. Large granule fraction washed once with 0.88 M sucrose	10		28 (3)
	20	63 (7)	
D. Large granule fraction washed twice with 0.9% NaCl	20	(A) 59 (3)‡ (B) 16 (6)	19 (3)
E. Washings from large granule fraction	20	31 (6)	33 (3)
F. Small granule fraction	10		105 (3)
	20	87 (6)	
G. Small granule fraction washed twice with 0.9% NaCl	20	15 (3)	
H. Washings from small granule fraction	10		100 (3)
	20	50 (3)	214 (3)
I. Supernatant liquid from centrifuging at 15,000 × g	10		97 (3)
	20	44 (8)	

* Amount of fraction equivalent to the milligrams of fresh tissue indicated.

† Figure in parentheses indicates number of rats used in assays.

‡ The high value for the first two groups of rats (A) is probably due to incomplete extraction of the granules since a smaller volume of sodium chloride solution was used than for the granules of the other groups of rats (B).

ventional Warburg apparatus according to the method used in this laboratory (McShan and Meyer[7]).

Results and discussion. Succinoxidase activity. The large granule fractions C obtained from two groups of normal rats contained approximately 60% of the total succinoxidase activity found in the whole homogenates. Fraction C consisted largely of mitochondria as shown by examination of stained smears. In this connection it is of interest that Schneider,[1] and Hogeboom, Claude, and Hotchkiss[2] reported that 70 and 74%, respectively, of the succinoxidase activity of rat liver is present in the large granule fraction. Hogeboom, Schneider, and Pallade[4] prepared rat liver homogenates in 0.88 M sucrose and recovered 65 to 82% of the succinoxidase activity in the mitochondria.

Gonadotrophin. The results obtained on assay of the various fractions of rat pituitary tissue are summarized in Table I. The results obtained with the whole homogenates designated as A in Table I show, as is already known, that rat pituitary glands contain a high level of gonadotrophin, and that the level of glands from castrate animals is greater than that of glands from normal rats. Very little gonadotrophic activity was retained in Fraction B, which consisted of various elements such as nuclei and connective tissue. These elements comprised a high percentage of the solid content of the glands.

The large granule fraction C consisted to a large extent of mitochondria as shown by the staining method of Bensley.[6] The small granule fraction F contained many fewer mitochondria than did fraction C. The results given in Table I show that both these fractions contained gonadotrophin, which is similar to the results obtained by Catchpole[8] who reported that a major part of the gonadotrophin of sheep pituitary glands is found in the large granule fraction.

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[7] McShan, W. H., and Meyer, R. K., *Arch. Biochem.*, 1946, **9**, 165.

The large granule fraction C plus the small granule fraction F of glands from both normal and castrate animals contained more than 50% of the total gonadotrophin. The major part of the remaining hormone is found in the soluble fraction I, which may be accounted for either on the basis of the presence of small granules which were not removed by centrifugation, or by the solubility of the hormone in the sucrose. The greater part of the gonadotrophin was removed from the granule fractions C and F by extraction with 0.9% sodium chloride solution to give fractions E and H, respectively. The insoluble fractions (D and G) that remained after extraction contained little gonadotrophin. Whether the gonadotrophin was extracted from the granules or whether granules containing gonadotrophin were dissolved is not clear from present results. Further work is necessary to determine whether the hormone is associated with the mitochondria or with granules that are recovered with the mitochondria.

Conclusions cannot be made as to whether the FSH and LH activities are differentially distributed between the different fractions since all the active fractions produced follicles and corpora lutea.

Summary. Approximately 60% of the succinoxidase activity of rat pituitary glands were found to be associated with the large granule fraction, and more than 50% of the gonadotrophin of these glands was associated with two granule fractions isolated from sucrose homogenates by differential centrifugation. The supernatant fraction from which the granules were removed contained the remaining gonadotrophic activity. The gonadotrophin can be extracted from the isolated granules with isotonic sodium chloride solution.

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[8] Catchpole, H. R., Abstract, *Fed. Proc.*, 1948, **7**, 19.

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17207. Urinary Excretion of Amino-acids During Alloxan-induced Diabetes in Rats.*

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The experiments reported here were undertaken to determine whether the metabolic disturbances of alloxan-induced diabetes in rats, with and without insulin-control, would entail an abnormal excretion of amino-acids in the urine.

Paper chromatographic methods developed by Consden and Martin[1] and adapted to urine analysis by Dent[2-4] have greatly facilitated the qualitative detection of amino-acids. Several investigators applying these methods have gathered much new information on the abnormal amino-acidurias found in the Fanconi syndrome,[2,3] acute yellow atrophy of the liver,[2] and in progressive muscular dystrophy.[5] Results of studies on these unrelated disorders indicate that the patterns of urinary

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* Aided by a grant from the Nutrition Foundation, Inc.

† Postdoctorate Research Fellow, National Institute of Health, U. S. Public Health Service.

[1] Consden, R., Gordon, A. H., and Martin, A. J. P., *Biochem. J.*, 1944, **38**, 224.

[2] Dent, C. E., Conference on Metabolic Aspects of Convalescence, Josiah Macy, Jr. Foundation, 1946, Nov. 12-13, 126.

[3] Dent, C. E., *Biochem. J.*, 1947, **41**, 240.

[4] Dent, C. E., *Biochem. J.*, 1948, **43**, 169.

[5] Ames, S. R., and Risley, H. A., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 131.

TABLE I.
Urinary Excretion of Amino-acid N and Glucose, and Balance N During Periods of Control and Insulin Insufficiency.*

Period	Balance N, mg	Urine amino-acid N mg	Urine amino-acid N % of total urine N	Urine glucose, g
Normal control	+57.3	.961	.6	0
Diabetic + insulin	+14.4	1.24	.5	.869
Diabetic no insulin	—126.	1.29	.6	2.25

* The values represent the average of 9-24 hr collections, 3 from each of 3 rats.

TABLE II.
Amino-acids and Related Substances in Urines of Rats, Demonstrated by Paper Chromatography.

Amino-acids	No. of 24 hr urine samples containing the respective amino-acids: Normal control (9 samples)	Alloxan-diabetes + insulin (9 samples)	Alloxan-diabetes without insulin (9 samples)
Aspartic acid	6	5	2
Glutamic "	9	9	9
Glycine	9	9	8
Alanine	9	9	9
Valine	3	3	1
Leucine	3	2	1
Taurine	6	[illegible]	9
Glutamine	9	7	8
Unidentified polypeptide	5	5	5

excretion of amino-acids may vary considerably in conditions that involve disturbances of nitrogen metabolism in the body and alterations in renal function. It seemed of interest to determine whether ketonemic acidosis in the diabetic rat, with greatly increased cellular catabolism and losses of nitrogen from the body, would be characterized by significant changes in amino-aciduria.

Methods. Male Rockland Farm rats weighing approximately 250 grams were fed the diet mixture of Cox and Imboden.[6] The diet was thoroughly mixed to ensure uniformity of composition, especially with regard to the nitrogen content of aliquots. Adequate supplements of vitamins A and D were given at weekly intervals. The animals were placed in individual metabolism cages designed to permit measurement of food intake and for separate collection of the urine and feces. Urine specimens were collected under toluol and preserved by freezing. Alloxan (Bios) was administered as a single dose, 50 mg per kg body weight, in 2.5% aqueous solution, injected intravenously after the rats were fasted 12 hours.

Total nitrogen determinations on diet, urine, and feces were performed by the micro-Kjeldahl method. Urea nitrogen was determined by the manometric method of Van Slyke and Kugel.[7] Amino-acid nitrogen was determined by the ninhydrin, carbondioxide method of Van Slyke, MacFadyen, and Hamilton.[8] The Nelson modification[9] of the Somogyi method was employed for the determination of glucose in blood and urine. Qualitative tests for acetone in the urine were made with a powdered mixture of sodium nitroprusside, sodium carbonate, and ammonium sulfate (Denco test). Paper chromatographic methods as described by Dent[2,4] were used to detect and identify individual amino-acids in all urines.

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[6] Cox, W. M., and Imboden, M., PROC. SOC. EXP. BIOL. AND MED., 1936, **34**, 443.

[7] Van Slyke, D. D., and Kugel, V. H., *J. Biol. Chem.*, 1933, **102**, 489.

[8] Van Slyke, D. D., MacFadyen, D. A., and Hamilton, P. B., *J. Biol. Chem.*, 1943, **150**, 251.

[9] Nelson, N., *J. Biol. Chem.*, 1944, **153**, 375.

Experimental. The results summarized here were obtained on 3 rats, each studied in 3 metabolic periods of 3 or more days: I, in normal state during preliminary control periods; II, in the diabetic state following the injection of alloxan, treated with adequate doses of insulin, and III, after withholding insulin. Severely diabetic states were induced in every animal, as indicated by the rapidity of the development of acetonuria, acidosis and death within 3 to 5 days when insulin was withheld. The preliminary period I included three consecutive 24-hour collections of urine. After the administration of alloxan the ensuing diabetic state was controlled by daily injections of 1 to 3 units of protamine zinc insulin, the doses being adjusted to maintain the body weight constant and to keep the urine acetone-free. After the establishment of satisfactory control, 24-hour collections of urine were resumed and continued through periods II and III, until the animal died.

Data from 3 rats are presented in Table I. The individual amino-acids identified by the chromatographic method in the urines of the 3 animals in respective periods are listed collectively in Table II.

Discussion. During periods of severe and fatal acidosis in the alloxan-diabetic rat, deprived of insulin, the pattern of excretion of amino-acids in the urine remained essentially unchanged, compared with periods of control. The ratio of amino-acid nitrogen to total nitrogen excreted in the urines remained relatively constant, independent of the development of ketosis, acidosis, and increased excretion of total nitrogen. These facts suggest the conclusion that although protein catabolism is increased in alloxan-induced diabetes during periods of insulin insufficiency, normal metabolic pathways are utilized, and amino-acids are efficiently conserved by the kidney. This is in accord with the results of studies on human diabetics as reported by Hall.[10]

Summary. Nitrogen balance studies on 3 male rats during periods of normal control, of alloxan-induced diabetes with insulin therapy, and of fatal acidosis after withholding insulin, are reported.

The pattern of excretion of urinary amino-acids, and the ratio of total amino-acid nitrogen to total urinary nitrogen remained essentially unchanged throughout the 3 periods, including the terminal stage with greatly increased losses of total nitrogen.

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[10] Hall, D. A., *Biochem. J. Proceedings*, 1948, **43**, lvii.

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17208. Lack of Identity of Hyaluronidase Inhibitor and Certain Mucoproteins in Blood Serum.*

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Winzler and co-workers[1,2] observed that elevations of plasma mucoprotein levels occur in patients with cancer and pneumonia. Prinzmetal *et al.*[3] have noted a similar increase in patients with myocardial infarctions.

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* Aided by a grant from the National Cancer Institute, U. S. Public Health Service, Bethesda, Md., and by the Committee on Growth of the National Research Council, acting for the American Cancer Society.

[1] Winzler, R. J., Devor, A. W., Mehl, J. W., and Smyth, I. M., *J. Clin. Invest.*, 1948, **27**, 609.

[2] Winzler, R. J., and Smyth, I. M., *J. Clin. Invest.*, 1948, **27**, 617.

[3] Simpkin, B., Bergman, H. C., and Prinzmetal, M., *Am. J. Med.*, 1949, in press.

Seibert *et al.*[4,5] have found that the carbohydrate content of human serum proteins increases in cancer and tuberculosis, and Niazi and State[6] have confirmed this elevation in cancer and infectious diseases. These findings are in accord with similar results reported in the earlier literature by other investigators. Meanwhile, it was shown by Glick and coworkers that a similar elevation in the level of hyaluronidase inhibitor (not the inhibitor that is an antibody to hyaluronidase) occurs in cancer,[7] and in a wide variety of both virus and bacterial infections.[8–11] Increases of hyaluronidase inhibitor in infectious diseases were also reported by Thompson,[12] Friou and Wenner,[13] and others.

It could reasonably be supposed that the hyaluronidase inhibitor might be associated with mucoprotein or carbohydrate compounds that could compete with hyaluronic acid for the enzyme. This possibility appeared to be strengthened by the electrophoretic studies of Winzler *et al.*,[1] which indicated that the isolated mucoprotein migrated nearer to the albumin fraction than to the other plasma proteins at pH 8.3, and of Glick and Moore,[14] which showed that the hyaluronidase inhibitor in the serum migrated chiefly with the albumin at pH 8.6.

These considerations, along with the observed parallelism in the elevation of both the inhibitor and the mucoprotein in the same diseases, prompted the present investigation of the possibility of their association or identity.

Mucoprotein samples of human serum prepared from perchloric acid filtrates by the procedure of Winzler *et al.*[1] showed little inhibitory activity per gram of protein compared to serum. Plasma mucoprotein preparations isolated by an ammonium sulfate procedure[15] which were electrophoretically homogeneous also had very little hyaluronidase inhibitor activity. The very low isoelectric points of plasma mucoproteins permit their electrophoretic separation from other plasma proteins at pH 4 or 4.5.[16,17] We have isolated plasma mucoproteins electrophoretically and tested them for hyaluronidase inhibitor. One such sample which was isolated, dialyzed, and lyophilized had somewhat more inhibitory effect but was still much weaker than fresh serum on an equal total protein basis. Adding magnesium ions to these preparations had no influence.

In view of the instability of the hyaluronidase inhibitor, it was not felt that its lack of identity with plasma mucoproteins was established by the foregoing results. The experiment shown in Table I, however, shows more conclusively that hyaluronidase inhibitor is not associated with the acidic plasma mucoproteins.

A pooled sample of serum freshly obtained from several patients with lobar pneumonia was adjusted to pH 4.5 with approximately 2 N acetic acid. A sample was removed for the hyaluronidase inhibitor determination, and the remainder of the serum was centrifuged to remove the small amount of precipitate which had formed. This clarified serum was then immediately subjected to electrophoresis, without any preliminary dialysis, against an acetate buffer in sodium chloride. The total acetate concentration was 0.02 M,

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[4] Seibert, F. B., Seibert, M. V., Atno, A. J., and Campbell, H. W., *J. Clin. Invest.*, 1947, **26**, 90.

[5] Seibert, F. B., Pfaff, M. L., and Seibert, M. V., *Arch. Biochem.*, 1948, **18**, 279.

[6] Niazi, S. A., and State, D., *Cancer Res.*, in press.

[7] Hakanson, E. Y., and Glick, D., *J. Nat. Cancer Inst.*, 1948, **9**, 129.

[8] Glick, D., and Gollan, F., *J. Inf. Dis.*, 1948, **83**, 200.

[9] Grais, M. L., and Glick, D., *J. Invest. Dermatol.*, 1948, **11**, 259.

[10] Glick, D., and Campbell, B., PROC. SOC. EXP. BIOL. AND MED., 1949, **70**, 29.

[11] Grais, M. L., and Glick, D., *J. Inf. Dis.*, in press.

[12] Thompson, R. T., *J. Lab. Clin. Med.*, 1948, **33**, 919.

[13] Friou, G. J., and Wenner, H. A., *J. Inf. Dis.*, 1947, **80**, 185.

[14] Glick, D., and Moore, D. H., *Arch. Biochem.*, 1948, **19**, 173.

[15] Weimer, H., Mehl, J. W., and Winzler, R. J., submitted for publication.

[16] Petermann, M. P., and Hogness, K. R., *Cancer*, 1948, **1**, 104.

[17] Mehl, J. W., Golden, F., and Winzler, R. J., submitted for publication.

TABLE I.
Hyaluronidase Inhibitor in Mucoprotein Concentrates.

Sample	mg % mucoprotein-tyrosine	% hyaluronidase inhibition per 0.02 ml
Normal serum	2.2	21.2
Pooled pneumonia serum frozen immediately	14.0	59
Same adjusted to pH 4.5 and frozen	12.0	11
Same adjusted to pH 4.5 centrifuged and supernatant frozen	11.8	8
Same adjusted to p.H 4.5 centrifuged and supernatant held in electrophoresis bath during experiment	13.3	5
Compartment I (acidic mucoproteins)	11.4	8
Compartment II (normal globulin and mucoprotein with reduced globulin)	10.5	13
Compartment III (globulins, no mucoprotein)	0.8	8
Compartment IV. Normal albumins and globulins (reduced mucoprotein)	6.7	6

the ionic strength 0.1, and the pH was 4.5 at 22°C. A 4-compartment cell was employed, and compensation was used as needed to keep the upper edge of the albumin boundary at the middle junction of the cell on the side towards the anode. The electrophoresis experiment was terminated when the fastest mucoprotein component had reached a point about 2/3 of the distance up the upper half of the anodic limb (compartment I). At the same time, the most rapidly moving globulin had reached the top of the upper half of the cathodic limb (compartment III).

At least 3 positively-charged components migrated toward the negative electrode—the fastest with a mobility corresponding to the major mucoprotein component. Compartment I contained only these acidic components and when removed, the concentration of these components should have been 1/2 to 2/3 that in the serum. Compartment II was the lower half of this arm and contained mucoprotein and albumin in the original concentration with somewhat reduced amounts of globulin. Compartment III was the upper section of the arm ascending to the negative electrode and contained the globulins and very little albumin or mucoprotein. Compartment IV was the lower section of this arm and contained globulins and albumin at original concentration with a reduced mucoprotein concentration. These samples were frozen immediately after the electrophoretic separation and were kept frozen until analysis of hyaluronidase inhibitor in order to prevent deterioration. Appropriate controls were also carried as is indicated in Table I. The mucoprotein levels were carried out using the tyrosine and carbohydrate determinations previously described[1] and the hyaluronidase inhibitor was determined as described earlier.[8,18] The data of Table I show that there is no correlation between the hyaluronidase inhibitor activity and the mucoprotein content of the various samples—the sample from compartment III, containing little or no acidic mucoprotein, having as much inhibitor as other compartments. The partial inactivation of the inhibitor resulting from adjustment to pH 4.5 does not seriously affect our conclusion that the hyaluronidase inhibitor and acidic plasma mucoproteins are not associated.

During the course of a study on serum from

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[18] Wattenberg, L., and Glick, D., *J. Biol. Chem.*, in press.

TABLE II.
Mucoprotein and Hyaluronidase Inhibitor Levels in "Lipoid Nephrosis."

Group of children	No.	Age range	Mean mucoprotein-tyrosine ± std. error of mean (mg %)	Mean % hyaluronidase inhibition ± std. error of mean per 0.02 ml fresh serum
Normal	40	1-15		21.2 ± 0.9
"	55	1-15	2.2 ± 0.1	
Nephrosis	9		1.5 ± 0.1	56.6 ± 3.7

TABLE III.
Hyaluronidase Inhibitor and Mucoprotein Levels in Miscellaneous Conditions.

Disease of child	Age	Mucoprotein-tyrosine (mg %)	% hyaluronidase inhibition per 0.02 ml fresh serum
Acute poliomyelitis	12	4.2	44
Acute lymphatic leukemia	8	9.4	29
Metastatic neuroblastema	1.3	9.8	56
Inactive rheumatic fever	16	5.1	11
Upper respiratory infection	11	2.6	31
Normal	5	3.8	23
"	12	2.6	33

children with nephrosis, which will be reported in detail elsewhere by Good, Kelley, and Glick, a further lack of correlation between the mucoprotein and inhibitor content of untreated sera was found (Table II). In this series the mucoprotein values measured by the method of Winzler *et al.*[1] were 32% less than the normal, while the inhibitor values were 167% greater than the normal. In other diseases, too, widely divergent serum values were found, as illustrated by a few examples given in Table III.

It is therefore clear that there is no identity of the hyaluronidase inhibitor and the acidic mucoproteins of blood serum as separated by electrophoresis at pH 4,5, by precipitation from perchloric acid filtrates, or by ammonium sulfate fractionation. This does not negate the possibility that the inhibitor may be found in other mucoprotein or glycoprotein components, nor is it at variance with the striking statistical correlation that has been observed between the elevation of serum mucoprotein and hyaluronidase inhibitor in cancer and infectious diseases.

Summary and conclusions. 1. Electrophoretically and chemically separated fractions of human serum containing high concentrations of mucoproteins showed no increase in non-specific hyaluronidase inhibitor when compared to native serum or other mucoprotein-poor fractions of serum.

2. In children suffering from "lipoid nephrosis" the hyaluronidase inhibitor levels of the serum were higher while the mucoprotein levels were significantly lower than normal.

3. In spite of the striking statistical correlation existing between the serum levels of mucoprotein and non-specific hyaluronidase inhibitor in a wide variety of human diseases, marked disparity in the serum levels of these substances occasionally occurs.

4. On the basis of these findings the lack of identity of the non-specific hyaluronidase inhibitor and these serum mucoproteins is pointed out.

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17209. Biotin-Carbohydrate Interrelationships in the Metabolism of *Leuconostoc.*[*]

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(Introduced by Emmett B. Carmichael.)
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During an investigation of the direct utilization of sucrose by *Leuconostoc*[1] it was found that the organisms apparently did not require biotin when the disaccharide was employed, but the presence of the vitamin was essential for growth when the constituent monosaccharides were used in the media. Because a variation in vitamin requirement as a function of carbohydrate source was not found reported in the literature, and because it appeared to offer the possibility of recognition of a new function of biotin, the observation was investigated further.

Experimental. The basal medium employed had the composition given in Table I. With the exception of the carbohydrate component, the basal medium was made up in 3/2 strength, distributed in 2 ml amounts in 10 × 100 mm test tubes closed with cotton plugs, and sterilized 5 minutes at 15 lb. pressure. The carbohydrate component was made up in triple strength, sterilized by Seitz filtration, and added, aseptically, in 1 ml volumes. If other additives were involved, *e.g.*, oleic acid or egg white, these were included in the carbohydrate solution so that the final volume per tube remained constant at 3 ml.

TABLE I.
Composition of Basal Medium.

Constituent	Conc. per liter g	mg
Carbohydrate	50	
Casein hydrolysate	5	
Cysteine		100
Tryptophane		100
Phenylalanine		50
Tyrosine		50
Adenine		10
Xanthine		10
Guanine		10
Thymine		10
Uracil		10
Nicotinic acid		1
Riboflavin		0.5
Thiamin chloride		0.5
Calcium pantothenate		0.5
Pyridoxine		0.4
p-Aminobenzoic acid		0.1
Folic acid		0.01
NaOAc	25	
NH_4Cl	5	
$MgSO_4 \cdot 7H_2O$		100
NaCl		10
$FeSO_4$		10
$MnSO_4$		10
KH_2PO_4		500
K_2HPO_4		500

Four sugar sources were employed: Sucrose, glucose, fructose and acid hydrolyzed sucrose. In the case of the first 3 sugars, the final concentration of 5% consisted of 90% of Seitz sterilized sugar and 10% of the same sugar sterilized in neutral phosphate buffer for 15 minutes at 15 lb. pressure. The resulting 0.5% concentration of heat sterilized sugar served as a source of the stimulatory materials reported to be necessary for the growth of many lactic acid organisms.[2] In the remaining instance sucrose was hydrolyzed with 0.1 N HCl under reflux for 80 minutes, neutralized with KOH and passed through a Seitz filter.

Three strains of *Leuconostoc* were used in this investigation: *L. mesenteroides* 683, *L. dextranicum elai,* and *L. dextranicum* 8086 (ATCC). Of the various strains available these were selected because of the wide variation in their respective dextran synthesizing capabilities. *L. dextranicum elai* consistently gives yields of dextran approximating complete conversion of the dextrose half of the sucrose molecule into the polysaccharide. The yields routinely obtained with *L. mesenteroides* 683 and *L. dextranicum* 8086 were, respectively, 65% and 33% of that theoretically possible from complete polymerization of the dextrose component.

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[*] Presented in part before the thirty-third annual meeting, Federation of American Societies for Experimental Biology, Detroit, April 1949.

[1] Carlson, W. W., and Whiteside-Carlson, V., *Fed. Proc.*, 1949, **8**, 189.

[2] Orla Jensen, S., *J. Soc. Chem. Ind.*, 1933, **52**, 374.

The inocula were prepared by incubating the cultures for 24 hours at room temperature in 5 ml of the complete basal medium (Table I) to which biotin was added in the low concentration of 0.01 μg per liter. The bacterial cells were centrifuged from the medium, resuspended in a double volume of sterile saline, recentrifuged and again resuspended in sterile saline to make a 1:12 dilution of the original culture. By means of a sterile syringe (20-gauge needle) one drop of this diluted inoculum was added to each 3 ml tube of medium. In the case of experiments involving *Leuconostoc* the tubes after inoculation were incubated for 72 hours at a constant temperature of 25° C.

As an organism requiring biotin *Lactobacillus arabinosus* 17-5 was also employed in this investigation for the purpose of comparison and assay. The culture was grown in the same basal medium and the inoculum was prepared in the same manner as described for the *Leuconostoc*. Each tube, however, was incubated at 37°C for 72 hours.

The rate of growth of all organisms was measured by titration of each tube of medium with standard alkali, bromthymol blue being used as indicator. The results, corrected for the blank titration of uninoculated controls, are expressed in terms of ml of 0.01 N acid produced per ml of culture.

Results and Discussion. The presence of biotin in sucrose has been reported by various investigators.[3,4] To verify the observation that *Leuconostoc* require biotin for growth in glucose and fructose media, but not in a sucrose medium, a sample of the disaccharide was hydrolyzed with acid to invert sugar. Kreuger and Peterson[5] have shown that autoclaving for 2 hours at 15 lbs pressure with 2 N acid did not destroy biotin added to media. It therefore seemed safe to assume that the conditions employed here, 80 minutes refluxing at atmospheric pressure with 0.1 N acid, would not destroy any biotin present in the sucrose samples.

The ability of the 3 strains of *Leuconostoc* to grow in media containing sucrose, or the same sucrose hydrolyzed by acid, was determined both in the presence and absence of added egg white. The results given in Table II show that whereas satisfactory growth occurred both in the sucrose and hydrolyzed sucrose media, added egg white was strongly inhibitory in the medium containing invert sugar. In contrast, in the sucrose medium, egg white inhibited growth only partially and then to a degree approximately inversely proportional to the yield of dextran. *L. dextranicum* 8086 with a dextran yield of 33%, was markedly inhibited by egg white, whereas *L. dextranicum* elai, which forms a theoretical yield of the polysaccharide, was insensitive to the biotin-binding action of egg white. *L. mesenteroides* 683 occupied a middle position both which respect to dextran yield and inhibition by egg white. Thus, to the degree that a strain of *Leuconostoc* utilizes sucrose via the mechanism resulting in dextran synthesis, it appears to be free of a requirement for biotin.

The ability of the *Leuconostoc* strains to grow in glucose or fructose media in the presence or absence of egg white also was determined. The low level of growth observed in the basal media without added biotin was suppressed by egg white in the cases of *L. dextranicum* 8086 and *L. mesenteroides* 683. Growth of *L. dextranicum* elai was increased by the added egg white. Shorb[6] has reported the presence of small amounts of vitamin B_{12} and the T. J. factor in egg white. In separate experiments, *L. dextranicum* elai was found to be stimulated slightly by vitamin B_{12}[†] and to respond strongly to tomato serum as a source of the T. J. factor. The observed stimulation by egg white thus appears to be due to its T. J. factor content.

Reversal of egg white inhibition by added biotin also was demonstrated. In order to

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[3] Jackson, W. R., and Macek, T. J., *Ind. Eng. Chem.*, 1944, **36**, 261.

[4] Hall, H. H., Paine, H. S., and Fabian, F. W., *Food Research*, 1947, **12**, 99.

[5] Kreuger, K. K., and Peterson, W. H., *J. Bact.*, 1948, **55**, 693.

[6] Shorb, M. S., *Science*, 1948, **107**, 397.

[†] We are indebted to Merck and Company, Rahway, N. J., for a sample of crystalline vitamin B_{12}.

TABLE II.
Effect of Egg White, Biotin and Oleic Acid on the Growth of *Leuconostoc* in Various Carbohydrate Media.

Additions to basal medium	Test organism	ml 0.01 N NaOH per ml medium			
		Sucrose	Hydrolyzed sucrose	Glucose	Fructose
None	*L. dextranicum* 8086	12.3	9.7	1.1	1.5
	L. mesenteroides 683	9.7	11.6	1.5	2.0
	L. dextranicum elai	7.2	5.8	0.7	0.3
Egg white 0.2 ml per tube	*L. dextranicum* 8086	2.4	0.7	0.1	0.1
	L. mesenteroides 683	5.7	0.4	0.6	0.4
	L. dextranicum elai	7.1	2.5	1.9	2.6
Egg white 0.2 ml per tube Biotin 0.01 μg per ml	*L. dextranicum* 8086	—	12.0	11.2	8.5
	L. mesenteroides 683	—	7.2	7.7	13.4
	L. dextranicum elai	—	7.3	3.5	7.8
Oleic acid 5 μg per ml	*L. dextranicum* 8086	11.6	8.4	4.0	2.5
	L. mesenteroides 683	11.9	11.3	4.7	6.2
	L. dextranicum elai	0.2	0.9	0.1	0.9
Egg white 0.2 ml per tube Oleic acid 5 μg per ml	*L. dextranicum* 8086	8.6	0.2	0.1	0.1
	L. mesenteroides 683	7.7	0.3	0.1	0.2
	L. dextranicum elai	1.9	2.0	0.2	0.9

eliminate variables such as activity of the inoculum, the data for Table II were all collected at one time. Tubes corresponding to the addition of both egg white and biotin to the sucrose medium were not included in this experiment. From results of other experiments, however, it can be stated that added biotin reverses such variable inhibition of growth in sucrose media as occurs with the various strains of *Leuconostoc*. Slight stimulation over the growth observed in the basal medium without added biotin was customarily found, traceable to the presence of stimulatory factors in egg white.

Oleic acid is regarded as a probable product of biotin activity and is stated to be capable of substituting for the vitamin in the case of certain lactic acid organisms.[7,8] The effect of oleic acid, at a level of 5 μg per ml, was determined for the various types of carbohydrate media given in Table II. *L. dextranicum* elai showed a marked sensitivity to oleic acid, growth being almost completely inhibited in all the media employed. With the other two strains of *Leuconostoc,* oleic acid produced little effect in sucrose or hydrolyzed sucrose media. Growth in glucose and fructose was stimulated, demonstrating an ability of the substance to partially replace the biotin requirement of the organisms. Reversal of egg white inhibition by added oleic acid occurred only with *L. mesenteroides* 683 and *L. dextranicum* 8086 in sucrose medium. In the case of *L. dextranicum* elai, the toxicity of oleic acid was reversed to a small extent by added egg white. Failure of oleic acid to reverse egg white inhibition in media containing invert sugar, glucose, or fructose, as compared with the result obtained in the sucrose medium, suggests that utilization of the intact disaccharide follows a different metabolic pathway.

The results given in Table II are taken to indicate that sucrose does contain traces of biotin, these small amounts of the vitamin being important to growth of the organisms in the invert sugar medium, and also in the sucrose medium to the extent that utilization of the disaccharide does not follow the path-

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[7] Williams, V. R., and Fieger, E. A., *J. Biol. Chem.*, 1946, **166**, 335.

[8] Boquist, H. P., and Snell, E. E., *J. Biol. Chem.*, 1948, **173**, 435.

TABLE III.
Assay of Biotin Level of Various Carbohydrate Media with *Lactobacillus arabinosus.*

Additions to basal medium	ml 0.01 N NaOH per ml medium			
	Sucrose	Hydrolyzed sucrose	Glucose	Fructose
None	1.4	4.3	1.7	0.7
Biotin 0.00005 μg per ml	4.6	8.8	4.0	5.2
Biotin 0.0005 μg per ml	19.0	23.3	23.3	16.6
Egg white 0.2 ml per tube	0.0	0.2	0.2	0.1
Egg white 0.2 ml per tube Biotin 0.01 μg per ml	23.3	25.2	26.7	20.1

way leading to polysaccharide synthesis.

An attempt was made to estimate the amount of the vitamin in the various carbohydrate media by means of a standard biotin assay organism, *Lactobacillus arabinosus* 17-5. The results, briefly summarized in Table III, show that only a low level of growth was obtained with any of the carbohydrate media. Egg white suppressed this low level of growth, complete reversal of the inhibition being observed with added vitamin. Added to the basal media at a level of 0.00005 μg per ml, biotin caused a definite stimulation of growth, a nearly maximal effect being obtained at a level of 0.0005 μg per ml. These results indicate that *Leuconostoc* respond to very low levels of biotin.

To test the possibility that *Leuconostoc* synthesize biotin, the cultures were grown in the sucrose and invert sugar basal media for 72 hours. The cultures were then sterilized for 15 minutes at 15 lb pressure and aliquots of the resulting solutions added to tubes of the glucose basal medium, after which they were inoculated with *L. arabinosus.* Representative titration figures, as obtained with the material from the growth of *L. mesenteroides* 683 in sucrose and invert sugar media were, respectively, 1.5 ml and 5.2 ml. Thompson[9] has reported that organisms that synthesize biotin release the vitamin into the medium. Since essentially no stimulation of the growth of *L. arabinosus* by *L. mesenteroides* culture solutions was observed, it appears unlikely that *Leuconostoc* synthesize biotin. It is therefore concluded that the observed difference in the biotin requirement of *Leuconostoc* in a sucrose medium, as compared with the requirement in media containing the constituent monosaccharides, is real. Information concerning the metabolic pathway in invert sugar, glucose, or fructose media, as compared with the pathway in a sucrose medium which leads to polysaccharide synthesis, thus may suggest a function for biotin in addition to those presently known.[10]

Summary. With 3 strains of *Leuconostoc* it was found that biotin was required for growth in media containing invert sugar, glucose, or fructose. In a sucrose medium the organisms were free of a biotin requirement to the degree that the disaccharide was utilized via the mechanism resulting in dextran synthesis.

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[9] Thompson, R. C., Univ. of Texas Publ. 4237, 1942, p. 87.

[10] Couch, J. R., Cravens, W. W., Elvehjem, C. A., and Halpin, J. G., *Arch. Biochem.*, 1949, **21**, 77.

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17210. Effect of Dihydroergocornine on the Heart.*

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In a previous study it was shown that 0.5 mg of ergotamine tartrate or dihydroergotamine 45 injected intravenously would cause inverted T waves in patients with organic heart disease to revert to normal.[1] Seven of the 19 patients studied developed these significant electrocardiographic changes under the influence of these drugs and in 5 patients severe anginal pain occurred. This effect was attributed to a direct vasoconstrictor action of the ergotamine preparations on the coronary arteries. The experiments were repeated after blockade of the autonomic ganglia with tetraethylammonium chloride. Six of the 10 patients who received ergotamine tartrate in addition to tetraethylammonium chloride developed normalization of the previously inverted T waves. Changes in the repolarization of the heart muscle induced by the coronary vasoconstriction were assumed to be responsible for these electrocardiographic changes.[2]

The present study was undertaken to show the influence of dihydroergocornine (DHO 180) on the electrocardiogram of patients with left ventricular hypertrophy secondary to hypertension or aortitis.

Material and Method. Of the 24 patients examined 6 had a history of anginal pain and 5 had syphilitic aortitis with marked electrocardiographic changes so that involvement of the coronary ostia could be assumed to exist. All patients had the pattern of left ventricular hypertrophy with a depressed RS-T segment and inverted T waves in lead I and high R waves with similar changes of RS-T and T in lead CF_5.

After 30 minutes of bed-rest a control electrocardiogram and the blood pressure were recorded. Then 0.5 mg of dihydroergocornine was injected intravenously and the blood pressure readings and the electrocardiogram were repeated after 1, 5, 15, and 30 minutes. Standard leads as well as CF_2 and CF_5 were taken.

Results. In all cases the electrocardiogram remained unchanged throughout the period of investigation. The position of the RS-T segment did not change and the inversion of the T waves remained the same (Table I). The blood pressure fell in most patients and this fall seemed to be more pronounced the higher the initial blood pressure was.

The constancy of the electrocardiographic pattern was even more remarkable since 11 of the 24 patients were 65 years old or older. Anginal pain did not appear even in those patients who had suffered from it before admission. No untoward symptom or reaction developed in any of the 24 patients. Careful observation did not reveal any changes apart from the change in the blood pressure.

Discussion. This investigation was undertaken because of the report that dihydroergocornine does not produce vasoconstriction in the sympathectomized limb and represents the first pure sympathicolytic derivative of ergot.[3] It belongs to the group of ergot alkaloids which combine a peripheral sympathicolytic action on the receptors in the vessels with a central nervous effect which diminishes vascular tone.[4]

In view of the recommendation of this drug for the treatment of peripheral vascular diseases and certain forms of hypertension[4] it seemed desirable to ascertain whether it promotes anginal pain and whether it has a constrictor effect on the coronary arteries. Our studies indicate that such effects are absent and that in contrast to ergotamine tartrate and dihydroergotamine tartrate the drug is

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* We are indebted to Dr. Henze of the Sandoz Chemical Company for the supply of DHO 180, used in this study.

[1] Scherf, D., and Schlachman, M., *Am. J. Med. Science*, 1948, **216**, 673.

[2] Schlachman, M., and Scherf, D., *Am. Heart J.*, 1949, in press.

[3] Bluntschli, H. J., and Goetz, R. H., *Am. Heart J.*, 1948, **35**, 873.

[4] Kappert, A., *Helvetica Med. Acta*, 1949, Suppl. 22.

TABLE I.
Changes of Blood Pressure and Electrocardiogram Due to Dihydroergocornine.

No.	Age	Sex	Diagnosis	Blood pressure			Electrocardiogram	
				Initial	5 min.	30 min.	Before	After
1*	57	F	Luetic aortitis	150/40	126/32	134/40	Left ventricular hypertrophy	No changes
2	60	F	Hypertensive cardiovascular Congestive heart failure	174/110	154/94	156/99	” ” ”	” ”
3*	64	F	Hypertensive cardiovascular Cerebral thrombosis	244/126	222/116	230/122	” ” ”	” ”
4	66	M	Generalized arteriosclerosis Diabetes mellitus Aortitis	190/86	172/78	156/82	” ” ” (early)	” ”
5	47	F	Hypertensive cardiovascular Cerebral thrombosis	248/134	234/128	210/120	Left ventricular hypertrophy	” ”
6	55	M	Hypertensive cardiovascular	168/138	146/114	150/116	” ” ”	” ”
7	62	M	Hypertensive cardiovascular Diabetes mellitus Pulmonary emphysema	172/96	144/88	138/88	” ” ”	” ”
8	42	M	Essential hypertension in malignant phase	184/136	160/126	162/120	” ” ”	” ”
9	32	M	Malignant hypertension	178/126	146/108	150/100	” ” ” PR 0.22	” ”
10	56	M	Hypertensive cardiovascular Luetic aortitis Congestive heart failure	234/132	186/112	184/112	Left ventricular hypertrophy PR 0.22	” ”
11	65	M	Hypertensive cardiovascular Carcinoma of urinary bladder	198/108	178/99	166/96	Left ventricular hypertrophy (early)	” ”
12	67	F	Hypertensive cardiovascular Arteriosclerosis	154/116	148/98	148/98	Left ventricular hypertrophy Multifocal extrasystoles	” ”
13	47	F	Luetic aortitis Congestive heart failure	252/104	204/90	198/92	Left ventricular hypertrophy PR 0.23	” ”

TABLE I. (continued).
Changes of Blood Pressure and Electrocardiogram Due to Dihydroergocornine.

No.	Age	Sex	Diagnosis	Blood pressure			Electrocardiogram	
				Initial	5 min.	30 min.	Before	After
14*	57	M	Hypertensive cardiovascular Old cerebrovascular accident with slight hemiparesis	228/118	198/104	186/102	Left ventricular hypertrophy	,, ,,
15	76	M	Hypertensive cardiovascular	228/94	194/80	204/92	,, ,, ,,	,, ,,
16	82	M	Hypertensive cardiovascular Cerebrovascular accident	186/112	144/86	134/88	,, ,, ,,	,, ,,
17	47	M	Essential hypertension	190/144	166/134	172/140	,, ,, ,,	,, ,,
18	74	M	Hypertensive cardiovascular Congestive heart failure	192/104	164/84	156/94	,, ,, ,, Auricular fibrillation, myocardial damage	,, ,,
19	72	F	Hypertensive cardiovascular Osteoarthritis	214/76	194/78	178/72	Left ventricular hypertrophy	,, ,,
20*	58	F	Hypertensive cardiovascular Osteoarthritis, obesity	174/90	152/86	166/84	,, ,, ,,	,, ,,
21	82	F	Hypertensive cardiovascular Arteriosclerotic cardiov. Possible thoracic aortic aneurysm	232/106	194/94	170/98	,, ,, ,,	,, ,,
22*	75	F	Generalized arteriosclerosis Coronary sclerosis	160/80	150/72	128/64	,, ,, ,,	,, ,,
23*	67	M	Coronary thrombosis (antero-lateral wall infarction)	140/86	130/80	118/80	,, ,, ,,	,, ,,
24	80	M	Hypertensive cardiovascular Arteriosclerotic cardiov.	154/56	136/52	126/54	,, ,, ,, Ventricular extrasystoles	,, ,,

* History of anginal pain.

not contraindicated in patients with coronary disease.

Summary. The intravenous administration of 0.5 mg of dihydroergocornine to 24 patients with left ventricular hypertrophy caused no electrocardiographic changes indicative of coronary vasoconstriction. Although 6 of the 24 patients suffered from anginal pain and many more certainly had coronary disease, no angina pectoris followed the injection.

This particular alkaloid of ergot seems to be a safe drug in patients with coronary artery disease.

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17211. Cultivation of Pseudorabies Virus in the Yolk Sac of the Developing Chick Embryo.

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Successful cultivation of pseudorabies on the chorioallantoic membrane of embryonated eggs has been achieved by a number of workers,[1-6] several of whom propagated the virus in serial transfer for more than 50 passages. They noted that the titer of virus increased with continued passage, ability to kill embryos was gained and a lengthened incubation period for laboratory animals was acquired. The virus, however, invariably produced fatal infection. Although no reports were found that related to the cultivation of pseudorabies virus by the yolk sac method of Cox,[7] the attenuation of rinderpest by Jenkins and Shope[8] and of Newcastle virus by Beach[9] prompted the hope that cultivation in this manner might modify pseudorabies virus also. In both the rinderpest and Newcastle studies attention was centered on virus obtained from fluids, chorioallantoic membranes and embryos while titers attained in yolk sacs were not mentioned specifically. Indeed, other than for rickettsial and psittacoid agents, no information could be found that covered the growth of the smaller viruses in the yolk sac. In the work that follows attention was focused on growth of pseudorabies in the yolk sac membranes and the consequent effects on the virus.

Technic. The pseudorabies virus used throughout this work was a Hungarian strain sent to us through the courtesy of Dr. R. E. Shope in an infected rabbit brain preserved in glycerol. Part of the infected rabbit brain after washing was ground with sterile saline to make a homogeneous suspension and then 0.2 cc was inoculated intracerebrally into a rabbit. Two days later and immediately after death of the animal with typical symptoms of the disease, the brain was removed aseptically, weighed and ground in saline with a glass grinder to make a 10% suspension. The suspension was distributed in vials, sealed, quickly frozen and stored under dry ice refrigeration. Virus stored under such conditions was used to inoculate eggs in the initial passage. In subsequent passages 10% suspensions of fresh yolk sacs, prepared as for the rabbit brain, were used except in the first

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[1] Mesrobeanu, I., *C. R. Soc. Biol.*, 1938, **127**, 1183.

[2] Bedenski, G., and Bruckner, I., *C. R. Soc. Biol.*, 1938, **129**, 406.

[3] Burnet, F. M., Lush, D., and Jackson, A. V., *Austral. J. Exp. Biol. and Med. Sc.*, 1939, **17**, 35.

[4] Glover, R. E., *Brit. J. Exp. Path.*, 1939, **20**, 150.

[5] Morril, C. C., and Graham, R., *Am. J. Vet. Res.*, 1941, **2**, 35.

[6] Bang, F. B., *J. Exp. Med.*, 1942, **76**, 263.

[7] Cox, H. R., *Pub. Hlth. Rep.*, 1939, **53**, 2241.

[8] Jenkins, Dubois L., and Shope, R. E., *Am. J. Vet. Res.*, 1946, **7**, 174.

[9] Beach, J. R., Bankowski, R. A., and Quortrup, E. R., *Cornell Vet.*, 1948, **38**, 341.

TABLE I.

Results in Experimental Animals Inoculated Intracerebrally with Various Dilutions of Pseudorabies Virus Cultivated in the Yolk Sac of Chick Embryos.

Test animals*	Yolk sac passage	Dilutions used†				
		10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Rabbits	0	—	1/1	2/3	0/2	—
	5	—	1/1	1/1	1/1	0/2
	12	—	1/1	1/1	1/1	0/1
	26	—	—	1/1	0/1	0/1
	40	—	2/2	1/1	0/1	—
	50	—	1/1	1/1	1/1	0/1
Guinea pigs	0	1/1	2/2	0/2	0/1	—
	5	1/1	3/3	3/3	0/2	—
	12	1/1	2/2	4/4	2/2	0/1
	26	1/1	1/1	1/1	0/1	0/1
	40	1/1	1/1	1/1	0/1	—
	50	1/1	1/1	1/1	1/1	0/1
Mice	0	3/3	12/12	2/6	0/3	—
	5	3/3	9/9	6/6	0/3	—
	12	3/3	6/6	9/9	5/6	1/3
	26	3/3	3/3	3/3	0/3	0/3
	40	—	3/3	3/3	0/3	—
	50	—	3/3	3/3	2/3	0/3

* .2 cc of the virus dilution inoculated in each rabbit and guinea pig and .05 cc in each mouse.

† Denominator indicates number of animals used; numerator indicates animals that showed pseudorabies.

TABLE II.

Results in Experimental Animals Inoculated Subcutaneously with Various Dilutions of Pseudorabies Virus Cultivated in the Yolk Sac of Chick Embryos.

Test animals*	Yolk sac passage	Dilutions used†							
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
Rabbits	0	—	—	—	2/2	2/2	0/1	—	—
	5	—	—	—	1/1	1/1	1/1	0/1	—
	12	—	—	—	1/1	1/1	1/1	1/1	0/1
	26	—	—	—	—	1/1	0/1	0/1	—
	40	—	—	1/1	1/1	1/1	0/1	—	—
	50	—	—	1/1	1/1	1/1	0/1	—	—
Guinea pigs	0	1/1	3/3	2/3	0/1	—	—	—	—
	5	—	2/2	3/3	3/3	0/1	—	—	—
	12	1/1	1/1	2/2	3/3	2/2	0/1	—	—
	26	1/1	1/1	1/1	0/1	0/1	0/1	0/1	—
	40	2/2	1/1	1/1	0/1	0/1	—	—	—
	50	2/2	1/1	1/1	0/1	0/1	—	—	—
Mice	0	—	3/3	2/3	0/6	0/3	0/3	—	—
	5	—	6/6	3/3	6/6	0/3	0/3	—	—
	12	—	3/3	3/3	6/6	0/3	0/3	—	—
	26	—	—	3/3	1/3	0/3	0/3	0/3	—
	40	3/3	3/3	3/3	0/3	—	—	—	—
	50	—	6/6	3/3	0/3	—	—	—	—

* 1 cc of the virus dilution inoculated in each rabbit and guinea pig and 0.5 cc in each mouse.

† Denominator indicates number of animals used; numerator indicates animals that showed pseudorabies.

few passages where this material was stored for 48 hours at 34°F. This condition of storage was found not to affect the titer of virus. In tests for sterility, samples from all inocula were placed on blood agar plates and blood agar slants. All showed no growth.

TABLE III.
Distribution of Pseudorabies Virus in Eggs Inoculated into Yolk Sac.

Material used	Yolk sac passage	Dilutions 10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Yolk sac	1	0	+	+	+	0
	14	0	+	+	+	—
	50	0	0	+	+	—
Chorioallantoic membrane	1	0	+	+	—	0
	14	0	+	+	—	0
	50	0	+	+	—	—
Chorioallantoic fluid	1	+	—	—	0	0
	14	0	+	+	+	—
	50	0	0	+	—	—
Embryo	1	+	—	—	0	0
	14	0	+	+	—	0
	50	0	+	+	—	—

+ Virus shown to be present when inoculated intracerebrally into guinea pigs.
—Virus not present.
0 Not done.

In attempts to cultivate pseudorabies virus in the yolk sac, 9 fertile hens' eggs that had been embryonated for 6 days were inoculated in each passage and 3 eggs were left uninoculated as a test of incubator conditions. After inoculation, all eggs were incubated at 37°C and thereafter candled twice daily to record any changes in the embryos. Immediately after death of the embryos, the amount of virus was determined in the following manner. Tenfold dilutions were made from culturally sterile 10% suspensions in chilled saline and held in iced water from the time of preparation to completion of inoculations. From each dilution, 0.2 cc was inoculated intracerebrally into each of 1 or more rabbits and guinea pigs, 1 cc subcutaneously into each of 1 or more rabbits or guinea pigs, 0.05 cc intracerebrally into each of a group of 3 or 12 mice, and 0.5 cc subcutaneously into each of a group of 3 or 6 mice. Animals were observed twice daily and signs of illness recorded. In animals that showed no signs of illness, it was found that the first inoculation had conferred no immunity.

Transfer of virus in serial passage. After 5, 12, 26, 40 and 50 serial passages, tests were made for the presence and content of virus in the yolk sac. These results in comparison with the virus passed in rabbits are shown in Tables I and II.

As can be seen in Tables I and II, it appeared that the virus was maintained in the yolk sac in serial transfer for 50 passages and that the titer remained unchanged or perhaps increased. As tested by intracerebral inoculation in mice, serum that neutralized a hundred infective doses of the virus passed in rabbits likewise neutralized a similar amount of virus transferred in eggs for 50 passages. This result permitted the conclusion that the death produced in rabbits, guinea pigs and mice with suspensions of yolk sac from eggs after 50 serial transfers was due to pseudorabies virus and not another agent encountered in passage.

Distribution of virus in the egg. After showing that pseudorabies virus infected yolk sacs, it became of interest to determine further distribution of the virus in eggs inoculated in this manner. Each of a group of 12 eggs was inoculated with a 10% suspension of brains from infected rabbits, another with a 10% suspension of yolk sacs from infected eggs that had been transferred serially for 13 passages, and a third group with a 10% suspension of yolk sacs from infected eggs transferred for 49 passages. Eggs inoculated with virus passed in rabbits were harvested 3 days after inoculation, while those in the serial passages were harvested after 40 hours. From these eggs chorioallantoic fluids, yolk sacs, embryos and chorioallantoic membranes were obtained separately, and with the excep-

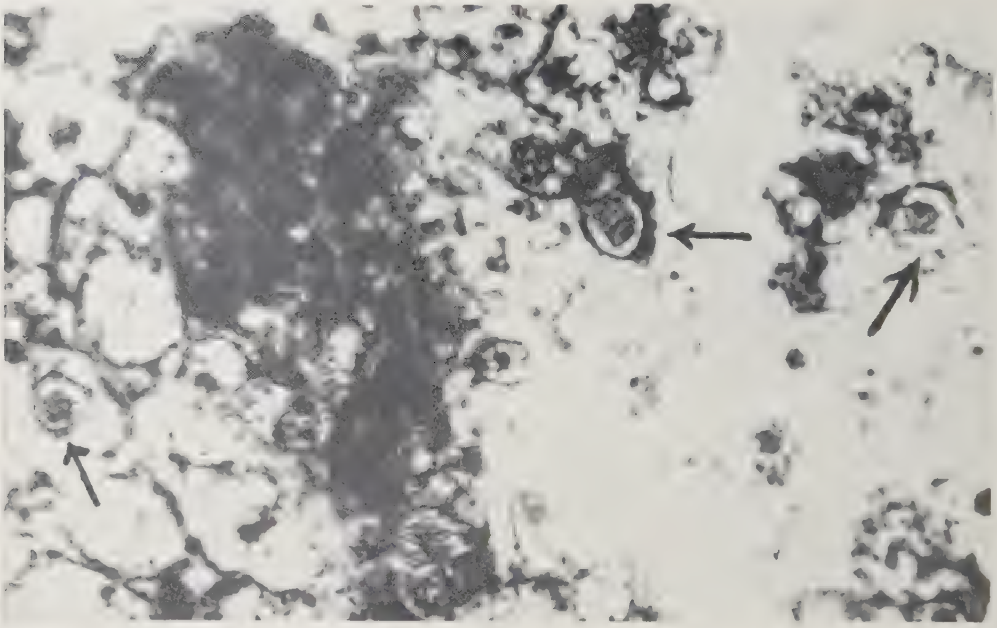

Fig. 1.
Yolk sac from an egg inoculated with virus transferred for 50 passages. Arrow indicates intranuclear inclusions.

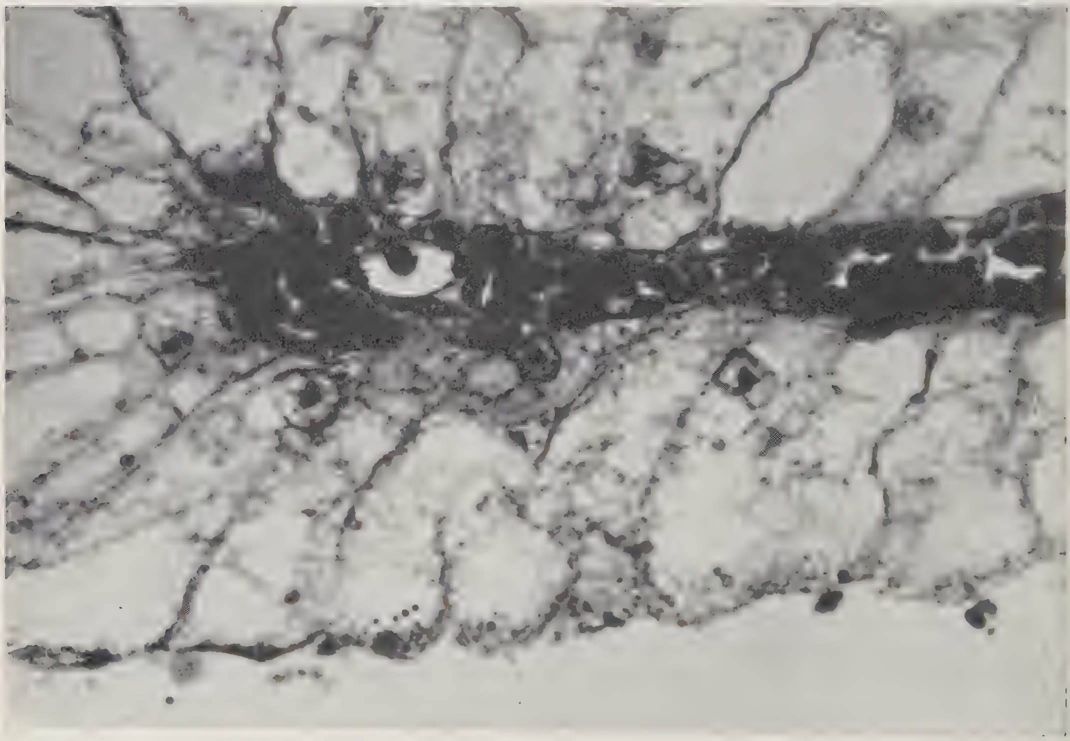

Fig. 2.
Yolk sac from an egg inoculated with saline, showing normal appearance of nuclei of yolk sac cells.

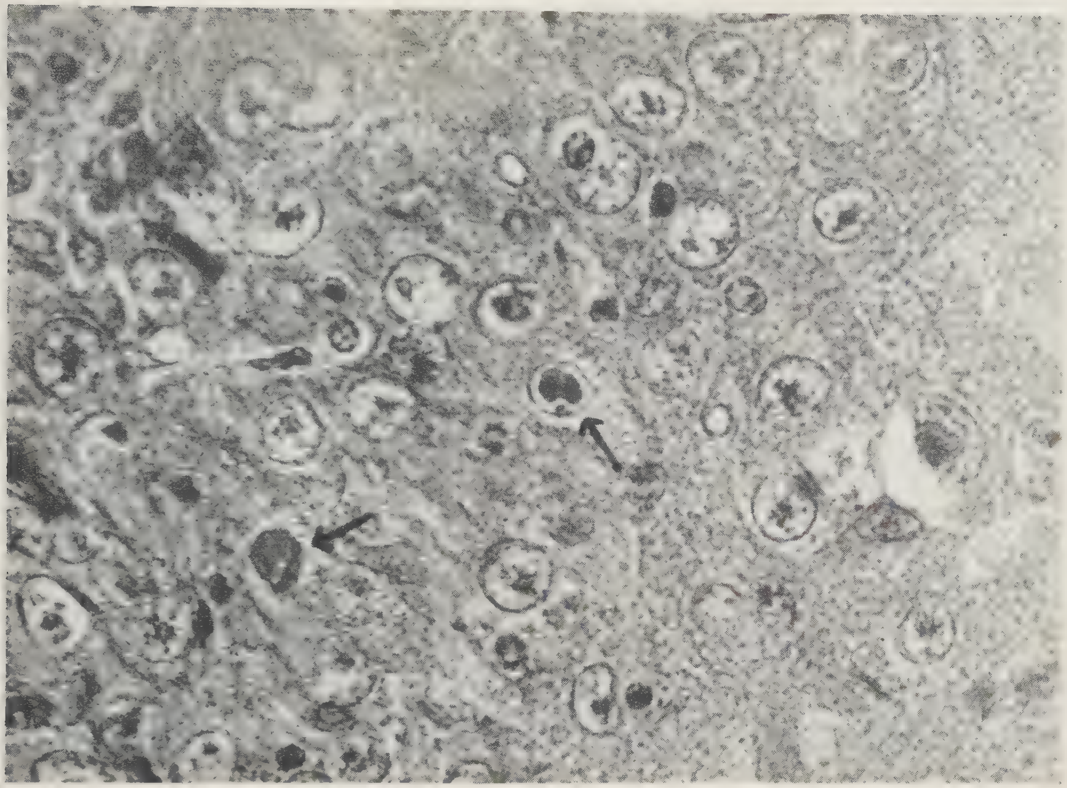

FIG. 3.
Brain from infected guinea pig inoculated intracerebrally with virus passed in rabbits. Arrow indicates inclusions in nuclei.

tion of chorioallantoic fluids, washed in 3 changes of saline, triturated and 10% suspensions made. Tenfold dilutions then were prepared from each suspension and tested in guinea pigs by intracerebral inoculations. The results are shown in Table III.

In Table III, it can be seen that the yolk sac always contained as much or more virus than other parts of infected eggs.

Effect of virus transferred in eggs on animals. In the first passage of pseudorabies virus into eggs, 60% of the embryos died 3 days after inoculation, in the 5th passage 80% of the embryos died within 48 hours, and in the 12th and subsequent passages all embryos died within a period of 40 hours.

In the rabbits, guinea pigs and mice inoculated both intracerebrally and subcutaneously with virus passed in rabbits and transferred 5, 12, 40 and 50 passages, comparisons were made of the effects produced by 10 to 100 lethal doses of virus. It was found that with continued serial transfer of virus in eggs, the interval of time between inoculation and death lengthened and animals survived longer after showing visible signs of illness. Moreover, a marked change in the signs of experimental disease was observed in inoculated animals. When rabbits were inoculated with virus passed in rabbits subcutaneously, characteristic skin signs and lesions in the form of an intense pruritus at the site of inoculation invariably developed. The skin became denuded of hair, abraded and infiltrated with serosanguinous fluid. Virus transferred in the yolk sac for 5, for 12, for 26 and for 40 passages produced similar signs, while animals that received virus transferred for 50 passages failed to develop signs or lesions in the skin. Instead, leg weakness developed 2 to 4 days before they died. Guinea pigs showed changes similar to rabbits except that the characteristic signs and lesions disappeared when the virus had been transferred for 40

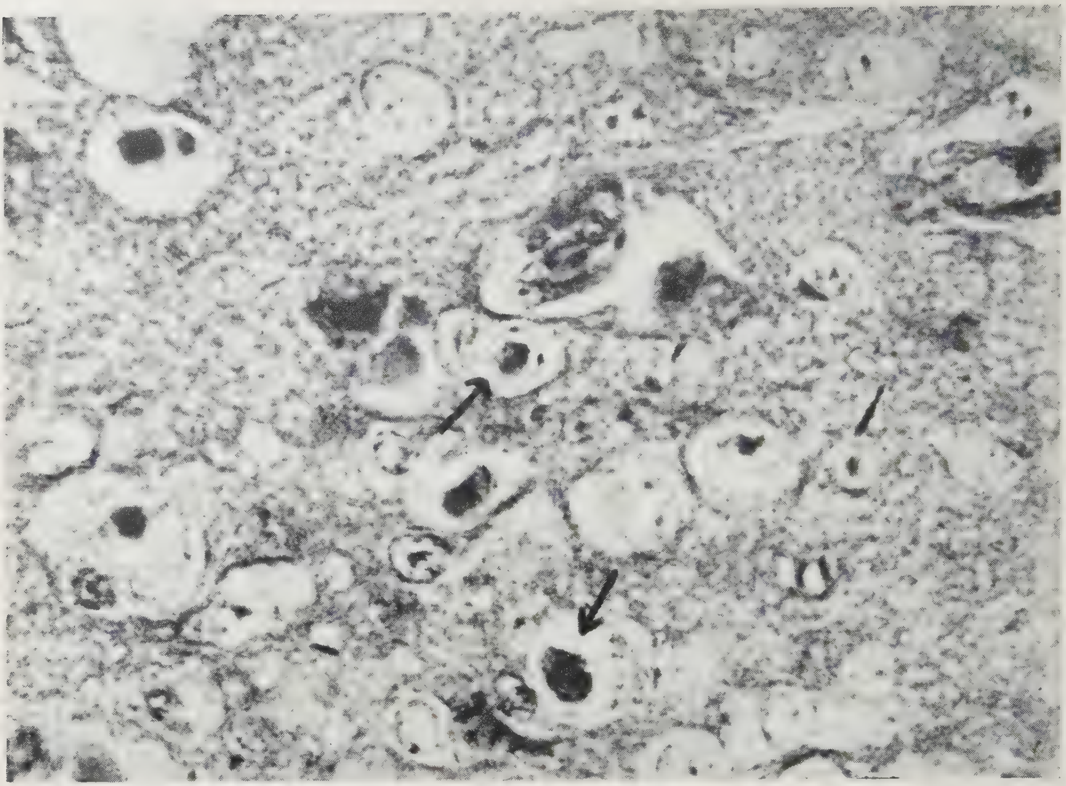

FIG. 4.
Brain from guinea pig inoculated intracerebrally with virus transferred for 50 serial passages in eggs. Arrow indicates inclusions.

passages in eggs. The animals then died with signs simulating those in guinea pigs inoculated intracerebrally. Mice that were inoculated subcutaneously showed paralytic-like signs when given virus transferred for 40 passages in eggs. Pruritus was not seen in mice even when inoculated with virus passed in rabbits.

Pathology. Embryos from the initial passage and each subsequent one were examined for lesions. In the first few passages, hemorrhages in the skin were seen over the brain region, but later hemorrhages extended over the entire surface of the embryos.

Brains of embryos from eggs inoculated with virus transferred for either 15 or 50 serial passages in eggs were prepared in Zenker's fixative and stained with hematoxylin and eosin and by Wilhite's method. Examination showed congestion of the superficial blood vessels with leucocytic infiltration throughout the brain. The neurons and neuroglial cells showed various stages of degeneration. Some of the mesothelial cells of the membranes covering the brain showed intranuclear inclusions in the form of homogeneous acidophilic material filling part or all of the nucleus. In brains of embryos from eggs inoculated with either rabbit brain virus or virus transferred for 5 passages, these changes were less pronounced and in some, only a slight congestion of the superficial blood vessels was found. Brains of embryos from eggs inoculated with saline showed no changes.

The most important changes seen in the cells of yolk sacs from eggs inoculated with virus transferred 50 passages were intranuclear inclusions (Fig. 1) that appeared as acidophilic homogeneous material filling most of the nucleus when completely formed. Margination of the chromatin along the inner surface of the nuclear membrane was seen. In the yolk sac cells of eggs inoculated with virus passed in rabbits and virus transferred for 5 passages in eggs, acidophilic small granules that were accumulated around the nucleolus was the only change observed. In the yolk sacs of eggs inoculated with saline, no changes were seen (Fig. 2).

Brains from guinea pigs inoculated intracerebrally with the rabbit brain virus and virus that had been transferred for 50 passages in eggs were fixed in Zenker's or formalin fixative and stained as for the egg material. In some brains from guinea pigs inoculated either with virus passed in rabbits or virus transferred for 50 serial passages in eggs, slight congestion was the only change observed, while in others, leucocytic infiltration and marked congestion of the blood vessels of the meninges and the superficial part of the brain were seen. Most of the animals showed the superficial nerve cells in different stages of degeneration. Intranuclear inclusions similar to those observed in the cells of yolk sacs from infected eggs were found in some nerve cells (Fig. 3 and 4).

Summary. Using the yolk sac method, a strain of pseudorabies virus has been cultivated in eggs for 50 serial transfers. That the virus multiplied in the yolk sac was shown in 4 ways: (1) suspensions of yolk sacs from infected eggs that had been transferred either for 5, 12, 26, 40 or 50 passages produced disease in rabbits, guinea pigs and mice; (2) the effects in animals of virus that had been transferred for 50 serial passages was neutralized by antiserum that neutralized virus passed in rabbits; (3) the yolk sac contained as much or more virus than either the chorioallantoic membranes, chorioallantoic fluid or embryos; (4) the virus produced characteristic intranuclear inclusions in the yolk sac cells like those seen in cells from infected animals.

With continued transfer of pseudorabies virus in eggs, embryos died 40 hours after inoculation while in the initial passage only 60% of the embryos died after 3 days. The capacity to invade certain parts of the egg such as the chorioallantoic fluids and embryos increased and more extensive changes were produced in the embryo. Continued passage also altered the effects on rabbits, guinea pigs and mice. The period of time from inoculation until death of animals lengthened, the local pruritus produced by subcutaneous inoculation of virus passed in rabbits no longer occurred and infected animals lived longer after showing signs of illness.

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17212. Antagonism of Choline and Heparin *In vivo* and *In vitro*.*

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Cabezas and Honorato[1] reported that heparin was inactivated by choline chloride. Various dilutions of heparin, choline and combinations of the 2 were added to fresh and 24-hour preserved plasma. The influence upon the prothrombin time was determined by the one stage Quick method.[2] These investigators did not draw final conclusions as to the mechanism of this effect, but suggested that there may be a chemical union of the choline with heparin, seroalbumin or albumin X.[3]

The annulation of heparin with prota-

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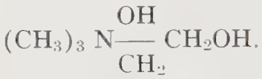
* This work was supported, in part, by the American Cancer Society on recommendation of the Committee on Growth of the National Research Council, and American Cancer Society Grant INSTR-23A.

† Rosalie B. Hite Postdoctoral Fellow.

[1] Cabezas, A., and Honorato, R., *Comunic. Society Biol. Santiago, Chile,* 1944, **2**, 26.

[2] Quick, A. J., *J. Am. Med. Assn.*, 1940, **119**, 118.

[3] Quick, A. J., The Hemorrhagic Diseases and the Physiology of Hemostasis. Thomas, Editor, 1942.

mine or toluidine blue is well established.[4–6] These latter compounds are complex molecules whereas choline is a simple molecule.  $(CH_3)_3\,\underset{CH_2}{\overset{OH}{N}}\text{—}CH_2OH$. In view of the recent work by Allen, Jacobson, Smith *et al.*, on the hemorrhagic syndrome produced by exposure to heavy doses of X-irradiation or nitrogen mustard,[7,8] the inactivation of heparin by choline becomes of considerable physiological and clinical interest. They have shown that this syndrome is due to an increase in the heparin or a heparin-like compound in the blood. Through 2 approaches *in vitro* and one approach *in vivo*, we have attempted to elucidate this problem.

Influence of choline and heparin upon the prothrombin time. A portion of Cabezas and Honorato's work was repeated as accurately as possible. The prothrombin time, Quick method,[2] was carried out on fresh whole human plasma. Choline chloride (2% and 4%) (Merck) in physiological saline was used in various volumes. Commercial heparin, 10 mg/cc (Roche-Organon) was diluted with physiological saline so that 0.025 cc equaled approximately 0.4 International Units and 0.025 cc equaled approximately 0.2 International Units. The commercial heparin contains approximately 110 International Units per mg. Rabbit brain thromboplastin (Maltine Co.) was used. The choline-heparin mixtures were not incubated.

Results. The prothrombin times on the control plasmas averaged 14.5 seconds; 0.2 U heparin added to the plasma showed an increase in the prothrombin time, averaging 4.9 seconds; 0.4 U heparin increased the prothrombin time an average of 13.7 seconds. Fifty γ, 2% choline, increased the prothrombin time an average of 1.0 seconds; 100 γ, 2% choline, increased the prothrombin time an average of 3.1 seconds; 100 γ, 4% choline, increased the prothrombin time an average of 2.9 seconds. Mixtures of heparin and choline in various concentrations and volumes added to the plasma always produced an increase in the prothrombin time over that of the heparin alone.

Interpretation. Our data illustrated no inactivation of the heparin by the choline. The slight increase in prothrombin time demonstrates the *in vitro* anticoagulant influence of choline. This confirms the observations of Zunz and LaBarre.[9]

When one analyzes Cabezas and Honorato's results, it is observed that the control prothrombin times were prolonged in most experiments. This is evidence of relatively inactive thromboplastin.

Absence of a chemical union of heparin and choline as measured by spectrophotometric determination. By the use of the Beckman spectrophotometer the absorption spectrum of a 0.1% solution of powdered heparin (Connaught 110 U/mg) dissolved in distilled water was determined. The peak of the absorption curve was found to be 265 mμ. Choline chloride in a 0.1% solution has no typical absorption bands. The two solutions were mixed in equal parts and the absorption curve was determined immediately, and after 6 hours of incubation.

Results. As may be seen in Fig. 1, there was no change in the absorption spectrum after heparin and choline were mixed.

Interpretation. If choline were combined with heparin there would be a definite shift in the spectrum of the choline-heparin mixture from that of the heparin alone. This demonstrates quite conclusively that heparin and choline do not enter into a chemical combination.

In vivo influence of heparin-choline on coagulation and prothrombin time. A series of experiments were designed to give *in vivo* evidence concerning the action of these substances on the coagulation mechanism. Three rabbits, each weighing 2.0 kg, were injected

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[4] Chargaff, L., and Olson, B., *J. Biol. Chem.*, 1937, **122**, 153.

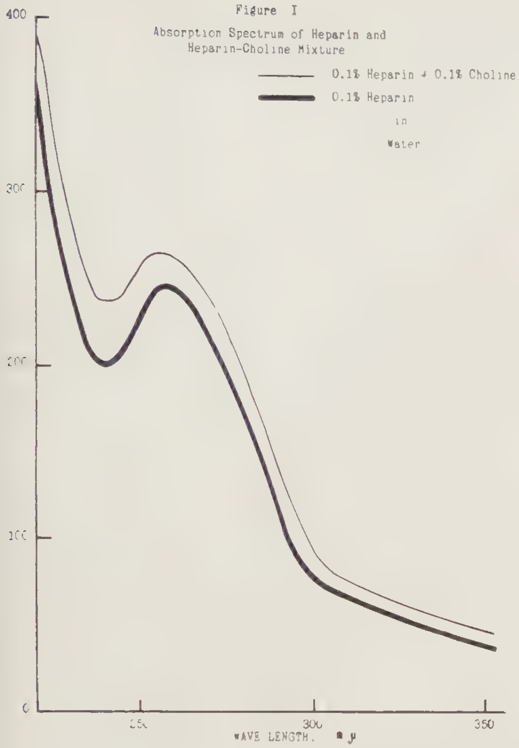
[5] Wilender, O., *Skand. Arch. and Physiol.*, 1938, **81**, Suppl. XV.

[6] Jorpes, J. E., Heparin in the Treatment of Thrombosis, Oxford University Press, 1946.

[7] Allen, J. G., and Jacobson, L. O., *Science*, 1947, **105**, 388.

[8] Smith, T. R., Jacobson, L. O., Spurr, C. L., Allen, T. G., and Block, M. H., *Science*, 1948, **107**, 474.

[9] Zunz, E., and LaBarre, J., *Compt. Rend. Biol.*, 1924, **90**, 655.



intravenously in an ear vein weekly with solutions of heparin and choline in the following ways:

1) 100 IU/kg heparin;
2) 10 mg/kg 2% choline chloride in saline;
3) 100 U/kg heparin followed immediately by 10 mg/kg choline;
4) 10 mg/kg choline followed in 5 min. by 100 U/kg heparin;
5) 100 U/kg heparin followed in 5 min. by 10 mg/kg of choline.

Blood was obtained by cardiocentesis with a 22 gauge needle. A specimen was drawn prior to and 10, 20, 30 and 60 minutes after injection. Coagulation times were done by a modification of the Lee-White method.[10] Blood in the amount of 0.5 cc was added to a 10 by 75 mm glass tube which had been rinsed with saline. The tube was tilted every 30 seconds and was kept at a temperature from 32 to 35°C. The end point was taken as the time the tube could be tilted vertically and the clot was solid. The prothrombin time was done by the one stage Quick method on whole plasma, and 0.9 cc of blood was added to 0.1 cc of 0.1 M sodium oxalate.

Results. Normal coagulation time varied between 3½ and 14 minutes. The choline alone had no remarkable effect on the blood coagulation other than possibly slightly accelerating it. Heparin alone, and heparin and choline in the various combinations, produced similar results.

The normal prothrombin times varied between 5.7 and 7.8 seconds and were not affected by the choline, whereas, they were slightly prolonged from 0.7 to 2.4 seconds after heparin alone, and 18.5 seconds after the heparin-choline injections. All prothrombin times were done in duplicate. If more than one second difference was obtained a third test was performed.

Interpretation. By means of the coagulation time and prothrombin time *in vivo,* choline has been shown to have no antagonistic influence on the heparin effect. One interesting observation is that choline alone consistently accelerated coagulation. This group of rabbits is too small, however, to draw definite conclusions as to the effect of choline on coagulation. Therefore, this is being studied further.

Summary. The previously reported antagonism of choline and heparin was studied *in vitro* and *in vivo.* When choline and heparin were mixed in whole plasma and the prothrombin time determined, the choline had no effect on the action of heparin. Choline alone slightly prolonged the prothrombin time. Choline and heparin do not combine chemically as shown by spectrophotometric determination. *In vivo,* choline has no effect on the action of heparin.

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[10] Lee, R. L., and White, P. D., *Am. J. Med. Sc.,* 1913, **145**, 495.

17213. Effects of Adrenocorticotrophic Hormone on Neuro-Muscular Function in Patients with Myasthenia Gravis.*

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The adrenocorticotrophic hormone of the pituitary gland has been administered to patients with myasthenia gravis mainly on the basis of the following observations and inferences: 1) the immediate cause of the symptoms of myasthenia gravis is a decrease of acetylcholine synthesis;[1-3] 2) administration of the adrenocorticotrophic hormone increases acetylcholine synthesis *in vivo*;[4] 3) increase of the lymphatic tissue (round-cell infiltration of various organs, mainly striated muscle[5] and "hyperfunctioning" thymus[6] have been found in patients with myasthenia gravis. Tissue fractionation studies[7,8] have shown that one of the sources of the substances that inhibit acetylcholine synthesis is the thymus. Administration of the adrenocorticotrophic hormone induces reduction in the mass of the thymus and the lymphatic tissue;[9,10] 4) removal of the pituitary gland in rats induces changes in the electromyogram[11] that closely resemble the abnormalities noted in patients with myasthenia gravis;[12,13] 5) the pituitary gland of several patients who died of myasthenia gravis showed accumulation of an eosinophilic colloid material suggesting altered function of the gland.[14-18]

This report aims to illuminate the nature of myasthenia gravis by a further analysis of its phenomenology. Therapeutic implications are outside its scope.

Material. Five patients with myasthenia gravis were women aged 24 (H.L.), 29 (J.R.), 31 (M.Y.), 37 (A.S.), and 45 (R.G.) years who had had myasthenia gravis for 4, 17, 10, 13, and 9 years, respectively. During the 3 years before this special study begun the patients experienced minor transient fluctuations but no long lasting or significant changes in their clinical states. They received a total of 300, 45, 180, 112, and 150 mg of neostigmine bromide a day, distributed over the waking hours, taken in from 3 to 6 hourly intervals. H.L. received also 75 mg of ephedrine sulfate, 3 g of potassium chloride, and 0.39 g of guanidine hydrochloride a day; R.G. also received 25 mg of ephedrine sulfate and 3 g of potassium chloride a day. The patients had had, in different degree of severity anorexia, general weakness, ptosis of the eyelids, weakness and easy fatiguability of the muscles of the palate, tongue, face, deglutition, arms, and legs.

Method. Various tests were performed

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* Published with permission of the Chief Medical Director, Departments of Medicine and Surgery, Veterans Administration, who assumes no responsibility for the opinions expressed or conclusions drawn by the authors.

[1] Torda, C., and Wolff, H. G., *Science*, 1943, **98**, 224.

[2] Torda, C., and Wolff, H. G., *J. Clin. Invest.*, 1944, **23**, 649.

[3] Torda, C., and Wolff, H. G., *Science*, 1944, **100**, 200.

[4] Torda, C., and Wolff, H. G., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 137.

[5] Weigert, C., *Neurol. Centralbl.*, 1901, **20**, 597.

[6] Sloan, H. H., Jr., *Surgery*, 1943, **13**, 154.

[7] Torda, C., and Wolff, H. G., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 69.

[8] Torda, C., and Wolff, H. G., *Am. J. Physiol.*, 1947, **148**, 417.

[9] Dougherty, T. F., and White, A., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 132.

[10] Simpson, M. E., Li, C. H., Reinhardt, W. O., and Evans, H. M., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 135.

[11] Torda, C., and Wolff, H. G., *Am. J. Physiol.*, 1949, **156**, 274.

[12] Torda, C., and Wolff, H. G., *Arch. Int. Med.*, 1947, **60**, 68.

[13] Harvey, A. M., and Masland, R. L., *Bull. Johns Hopkins Hosp.*, 1941, **69**, 1.

[14] Buzzard, E. F., *Brain*, 1905, **28**, 438.

[15] Tilney, F., *Neurographs*, 1907, **1**, 20.

[16] Marinesco, G., *Semaine Med.*, 1908, **28**, 421.

[17] Cavallero, C., *Tumori*, Ser. 2, 1944, **20**, 127.

[18] Torda, C., and Wolff, H. G., in press.

with the patients with myasthenia gravis for one week. Thereafter, the patients were administered intramuscularly adrenocorticotrophic hormone in amounts of 20 mg every 6 hours for 5 days. Tests were performed during the administration of the hormone, for 4 days thereafter, and at biweekly intervals for another 12 weeks. The following tests were performed.

Electromyography. Muscle action potential records were taken during repetitive indirect percutaneous stimulation of the ulnar nerve by 10 and 30 pulses per second for 30 seconds, with a stimulus of supramaximal intensity and of one millisecond duration. The method was described by Harvey and Masland[13] and Torda and Wolff.[12] The records were taken daily at the same time of the day, 3 hours after administration of neostigmine bromide during the control period and the period of administration of the hormone, and from 6 to 15 hours after administration of neostigmine bromide thereafter.

Myography. Muscle function was also tested by an ergograph immediately after completion of the electromyograms. The ergograph consisted of a heavy spring attached to an isotonic lever writing on a kymograph. The spring was stretched 2.5 cm once a second by the index and middle finger of the right hand exerting a tension of 15 kg.

Acetylcholine synthesis. Acetylcholine synthesis in the presence of the blood serum was studied following the method described by Torda and Wolff.[2] The method consists of incubation of a tissue containing choline acetylase with blood serum and determination of the amount of acetylcholine formed during the period of incubation. The amount of acetylcholine synthesized in the presence of serum from healthy controls averaged 2.08 μg ± 7% per 100 mg tissue containing the enzyme.

Results. During the 5 days of administration of ACTH all patients experienced a gradually increasing disability lasting until the second day after the completion of the injections. Thereafter, the patients exhibited increased well being, they began to reduce the daily intake of neostigmine bromide spontaneously (H.L. from 300 mg to 45 mg; M.Y. from 150 mg to 15 mg; A.S. from 112 mg to 15 mg; R.G. from 180 mg to 90 mg; and J.R. from 45 mg to 15 mg) and omitted the other medications. A partial remission of the symptoms occurred in all instances manifesting itself in marked decrease of muscle weakness, the easy fatiguability, and the anorexia. The ptosis of the eyelids, the weakness and easy fatiguability of the muscles of palate, tongue, face, deglutition, arms, and legs became less evident. However, the remission was incomplete, the muscle groups most severely involved in each patient showed only a partial recovery, as is to be expected after a muscle dysfunction of several years duration. Though not clearly definable, it is probable that there is some dwindling of the improvement in muscle function with the lapse of time. This incomplete remission persisted from the completion of administration of the hormone to the writing of this report (approximately 3 months).

Electromyography. Healthy subjects maintained the muscle action potential unaltered during repetitive indirect stimulation with 10 pulses per second for 30 seconds (Fig. 1A) and nearly unaltered (average decrease of 12%) during stimulation with 30 pulses per second for 30 seconds.[12,13] Before administration of the hormone patients H.L., M.Y., A.S., and R.G. exhibited the known decline of the amplitude of muscle action potential during repetitive indirect stimulation. The decline of the amplitude exceeded in all instances 35% on stimulation with 10 pulses per second for 30 seconds and 55% on stimulation with 10 pulses per second for 30 seconds (Fig. 1B-1E). During the period of administration of the hormone the abnormalities noted in the electromyograms of the patients gradually decreased. After completion of administration of the hormone and while on reduced medication the patients were so altered as to maintain the amplitude of action potential during repetitive indirect stimulation in a manner resembling that of healthy subjects. Patient J.R. had before and after administration of the hormone a normal electromyogram (Fig. 1F) as commonly occurs in patients mildly to moderately ill with myasthenia gravis. The electromyograms re-

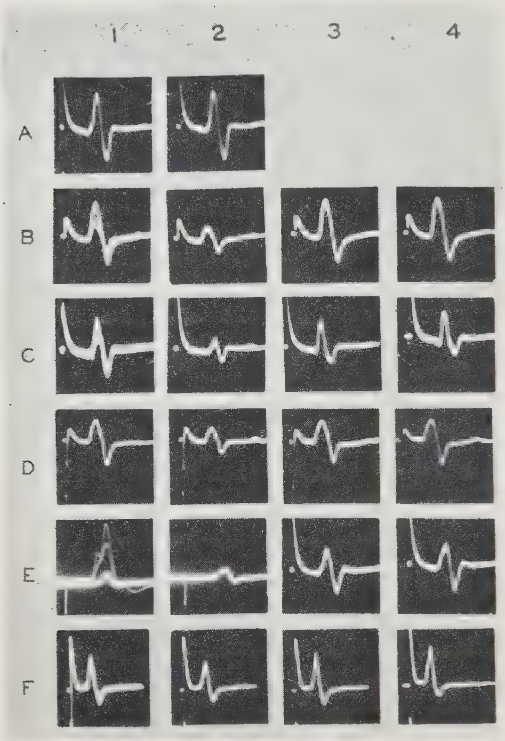

Fig. 1.

Effect of adrenocorticotrophic hormone on muscle action potential during repetitive indirect percutaneous stimulation of the ulnar nerve with 10 pulses per second.

Column 1 represents action potential records taken during the first few pulses of a 30 second stimulation period. Column 2 represents records taken at the end of the 30 second stimulation period. Column 3 represents action potential records taken during the first few pulses of a 30 second stimulation period the third day after completion of the series of administrations of the hormone. Column 4 represents records taken at the end of the 30 second stimulation period. Records in column 1 are comparable with records in column 2 of the same line and records in column 3 are comparable with those of column 4 of the same line since the position of the electrodes remained unchanged during the 30 second stimulation period. Records in column 3 are not comparable with those in column 1 of the same line since the recording electrodes could not be placed in exactly the same position on successive days. In all records the sweep circuit of the oscilloscope was synchronized with the stimulator so that successive stimuli and muscle action potentials were superimposed on the screen of the cathode ray tube. Group A contains records taken from a healthy control; group B records from patient H.L., group C records from M.Y., group D records from A.S., group E records from R.G., and group F records from J.R.

mained normal up to the writing of the present report (approximately 3 months).

Myography. The 10 control women stretched the spring on the average 150 times before occurrence of fatigue. Before administration of the hormone the patients with myasthenia gravis the average number of contractions (10 exper.) before occurrence of fatigue were 31 (R.G.); 44 (A.S.); 47 (M.Y.); 75 (H.L.), and 110 (J.R.). During the period of administration of the hormone the number of contractions slightly and gradually increased. After completion of administration of the hormone the work performance continued to increase and reached the fifth day the maximum. This was 105 (R.G.); 120 (A.S.); 180 (M.Y.); 250 (H.L.); and 175 (J.R.). Although this test involves motivation, such sudden and dramatic increase in performance suggests an improvement of the function of the neuromuscular system *per se.* The improved work performance persisted for the past 3 months.

Acetylcholine synthesis. In the presence of serum of the patients with myasthenia gravis the synthesis of acetylcholine as compared to healthy control subjects decreased by 55 (R.G.); 50 (H.L.); 42 (M.Y.); 40 (A.S.), and 25 (J.R.) %. In the presence of blood serum taken the third day after completion of administration of the hormone and biweekly thereafter during the past three months the ability of serum to support acetylcholine synthesis approximated normal values. Decreased acetylcholine synthesis in the presence of serum is specific for myasthenia gravis. The more severe the myasthenia gravis the less well the serum supports the activity of choline acetylase.

Comment. Administration of adrenocorticotrophic hormone to patients with myasthenia gravis was first begun by Torda and Wolff.[4,19] Beginnings of remission were observed in the 2 patients treated in the New York Hospital. Because satisfactory objective procedures testing neuromuscular function *per se* had not been elaborated in this laboratory at the time inferences concerning the effect of the hormone on patients with myasthenia gravis were deferred. A remis-

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[19] Torda, C., and Wolff, H. G., unpublished data, also Records of New York Hospital.

sion of the symptoms of myasthenia gravis in a moderately ill woman after administration of the hormone was reported by Soffer and collaborators[20] without application of objective testing procedures. The transitory impairment observed during the period of administration of the hormone was also observed by Hellman.[21] The increased general disability during the administration of the hormone may be due to changes in electrolyte distribution,[25] and in various metabolic processes (increase of secretion of some steroid hormone,[22–26] adverse effect on carbohydrate metabolism[25] and decrease of glutathione content of blood.[22]) These processes apparently offset the gradually improving function of the neuromuscular apparatus *per se*.[19]

The above investigations indicate that administration of the adrenocorticotrophic hormone induces changes suggesting the beginnings of an incomplete remission in patients with myasthenia gravis. If the view suggested by this laboratory[1–3] be valid, *i.e.*, that the immediate cause of the symptoms of patients with myasthenia gravis is a decrease in the synthesis of acetylcholine, then the observation that administration of the hormone increases the synthesis of acetylcholine[4] becomes extremely pertinent to an understanding of the apparent remission of symptoms observed in patients with myasthenia gravis after administration of the adrenocorticotrophic hormone. Because of its long lasting effects, it is inferred that the adrenocorticotrophic hormone acts upon some basic regulatory mechanism.

Summary. 1. Five patients moderately to severely ill with myasthenia gravis received 400 mg adrenocorticotrophic hormone given in amounts of 20 mg every 6 hours.

2. Changes that may indicate the beginnings of an incomplete remission occurred after completion of the injections. These changes consisted of decrease of the symptoms and outward manifestations of muscle dysfunction, disappearance of the abnormalities noted in the electromyogram, increased work performance on the ergograph, and increase to normal of the ability of serum to support acetylcholine synthesis. The incomplete remission is long lasting.

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[20] Soffer, I. J., Gabrilove, J. L., Laqueur, H. P., Volterra, M., Jacobs, M. D., and Sussman, M. L., *J. Mount Sinai Hosp.*, 1948, **15**, 73.

[21] Hellman, L., *Fed. Proc.*, 1949, **8**, 72.

[22] Conn, J. W., Louis, L. H., and Johnston, M. W., *J. Lab. Clin. Med.*, 1949, **34**, 255.

[23] Forsham, P. H., Thorn, G. W., Prunty, F. T. C., and Hills, A. G., *J. Clin. Endocr.*, 1949, **8**, 15.

[24] Thorn, G. W., Prunty, F. T. G., and Forsham, P. H., *J. Clin. Endocr.*, 1947, **7**, 459.

[25] Conferences of Macy Foundation, Metabolic Aspects of Convalescence, 16 Meeting, Oct. 27, 1947; 17 Meeting, Spring, 1948.

[26] Mason, H. L., Power, M. H., Rynearson, E. H., Letizia, C., Ciaramelli, M. D., Li, C. H., and Evans, H. M., *J. Clin. Endocr.*, 1948, **8**, 1.

17214. Alkaline and Acid Phosphatase Activity of the Embryonic Chick Retina.

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Evidence has been accumulating which indicates that the phosphomonoesterases may be associated in some way with the differentiation of embryonic tissue. Moog[1] has demonstrated that the common alkaline and acid phosphomonoesterase activity increases considerably during early embryonic development and reaches its maximum in whole chick embryo homogenates by the 10th day of incubation. From these and previous observations (Moog[2]), it appears that these enzymes enter in some way into the chemistry of differentiation of tissue. Although the above findings are very convincing, nevertheless attention should be directed to the fact that the results were obtained from tissue which was not only differentiating, but also undergoing rapid growth. This is a complicating factor and does not entirely eliminate the possibility that these phosphatases may be concerned with growth rather than differentiation. It seemed to the author that this concept could be further tested by using a single tissue which could be followed through its embryonic development from the time it begins to differentiate until it becomes functional. One tissue in the chick embryo which lends itself well to such a study is the retina. According to Weysee and Burgess[3] differentiation of retinal cells begins about the 12th day of incubation and is complete shortly before hatching.

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[1] Moog, F., *J. Cell. and Comp. Physiol.*, 1946, **28**, 197.

The present study was designed to determine whether any relationship exists between the stages of differentiation and the phosphatase activity of the embryonic chick retina.

Material and methods. New Hampshire Red chick embryos were used throughout this study. The eggs were obtained from a local hatchery and incubated in a commercial type incubator at a constant temperature of 102°F and a humidity of 90%. The retinas were removed from the eyes of 3 embryos of the desired age, blotted to remove excess moisture and weighed. The tissue was ground in a high speed micro grinder* to a uniform homogenate. The homogenate was then diluted with unbuffered saline solution (Krebs Ringer solution containing approximately .03M $MgSO_4$) so that 1 ml contained from 40-50 mg of fresh tissue. The substrate was combined with the buffer as follows: Alkaline mixture (pH 9.1) 1.09 g disodium phenylphosphate and 10.0 g of sodium barbital per liter. Acid mixture (pH 5.0) 1.09 g disodium phenylphosphate dissolved in 800 ml of a 0.2 N sodium acetate and 200 ml 0.2 N acetic acid. The enzyme activity was measured by a method modified from Moog[1] as follows: All experiments were carried out in test tubes (17 × 150 mm) containing 0.4 ml of substrate mixture. After pre-heating for 5 minutes in a bath kept constant at 37.5°C, 0.1 ml of homogenate was added to each tube. The preparations were then shaken for 20 minutes and the reaction stopped by adding 4.5 ml of 10% trichloroacetic acid. The precipitate was removed by filtration and 3 ml of the clear solution was analyzed for inorganic phosphorus by the method of Fiske and SubbaRow.[4] Ten minutes were permitted for the blue color to develop and all measurements were made with a Coleman spectrophotometer, calibrated with a series of standard KH_2PO_4 solutions. Experiments were always run in triplicate against 2 control tubes taken at zero time. Control values were subtracted from experimental values.

Results and discussion. The present study was confined to the retina from embryos beginning with the 12th day of incubation and ending approximately 3 days after hatching. This is the period during which the neural elements of the retina undergo maturation and differentiation into the various types of cells that characterize the functional organ. Although differentiation is very extensive during this time there is no appreciable change in the weight of the retina. The practice of using the weight of the tissue for computing data on chemical studies in embryonic tissues during different stages of development is sometimes considered a questionable procedure due to the changing concentration of the cytoplasm. However, since the chick retina did not increase more than 10% in weight over the period studied it seemed that there could be no serious objection in this case. The phosphatase activity is therefore expressed in terms of micrograms of inorganic

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[2] Moog, F., *Biol. Bull.*, 1944, **86**, 51.

[3] Weysee, A. W., Burgess, W. S., *Am. Nat.*, 1906, **40**, 611.

* The homogenizer is one of our own design consisting of a stainless steel drill driven by a high speed motor.

[4] Fiske and SubbaRow, *J. Biol. Chem.*, 1925, **66**, 375.

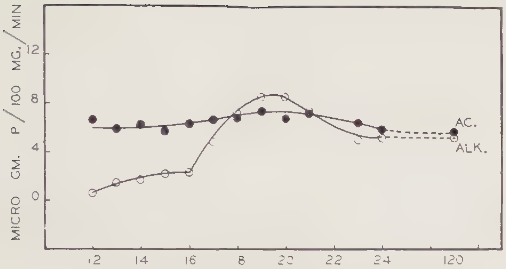

Fig. 1.

Alkaline (ALK) and acid phosphatase activity of the developing chick embryo retina.

phosphate liberated per 100 mg of fresh tissue per minute.

Fig. 1 depicts graphically the data obtained for both the alkaline (ALK) and acid (AC) phosphatase activity of whole homogenates of chick embryo retinas beginning with the 12th day of incubation and ending 3 days after hatching. The points on the curves represent means of data obtained from 3 separate series of embryos, incubated under identical conditions. It will be noted that the alkaline phosphatase activity at 12 days is almost negligible but rises gradually up to 16 days of incubation. Beginning with 16 days the activity increases rapidly up to 19 days where it reaches a maximum which is maintained through the 20th day after which it begins to fall at a rate comparable to the rise. However, about 3 days after hatching it levels off and maintains a rather constant rate.

The activity of the acid phosphatase, on the other hand, is relatively high at 12 days of incubation and does not appear to show any rise until about the 15th day. At this time there is a very small rise up to 19 days when it also decreases and eventually levels off at about 24 days. The broken lines on the graph designate the level of enzyme activity between the 24th and 120th day. Weekly analyses made between these ages showed no appreciable fluctuation in the enzyme activity and it is assumed that no further change took place.

It is interesting to note that the peak of both alkaline and acid phosphatase activity is reached on about the 19th-20th day of incubation. That the retina is differentiated at this time was demonstrated by the author,[5] when it was shown that 18 days after the beginning of incubation is the earliest stage in the development of the eye at which the pupillary constrictor reflex could be elicited by light stimuli.

Since these 2 events, namely completion of differentiation and development of maximum phosphatase activity correspond so closely it would appear that these enzymes may be concerned in some way with the morphogenesis of the retinal elements. These results are of interest therefore in the light of the findings of Moog, who demonstrated strikingly similar increases in both the alkaline and acid phosphatase activity of homogenates of whole chick embryos between the 2nd and 12th day of incubation during which time it was assumed that the tissues were undergoing differentiation. Thus the chick retina, which is known to differentiate between the 12th day of incubation and hatching time, lends further support to the concept that certain phosphomonoesterases may enter into the histochemistry of differentiation of embryonic tissues.

Summary and conclusion. 1. The alkaline and acid phosphatase activity was determined on whole homogenates of embryonic chick retina from the 12th day of incubation until 3 days after hatching.

2. The alkaline phosphatase activity increases slowly up to 16 days and then more rapidly to 19 days, when it reaches a maximum. After the 20th day it begins to decrease until 3 days after hatching, at which time it establishes a level of activity which is apparently constant throughout the life of the chicken.

3. The acid phosphatase activity is considerably higher than the alkaline at 12 days, but shows only a slight increase up to 19 days, after which it also falls to a new level.

4. Since the rise in activity of both acid and alkaline phosphatase correspond closely to the period of cellular maturation in the chick retina, this seems to give added support to the concept that these enzymes may be associated with the histochemistry of differentiation.

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[5] Lindeman, V. F., *Am. J. Physiol.*, 1947, **148**, 40.

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17215. Infection as a Factor Causing Death in the Eviscerate Rat.

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The data of these experiments indicate that the extent of bacterial contamination during the procedure of evisceration in the rat is an important factor in determining the survival time. Survival was not affected by the administration of gelatin or of Tyrode's solution.

Methods. Male rats of the Sprague-Dawley strain were maintained on a diet of Archer Dog Pellets. The technic of evisceration, which is done in 2 stages, has been described by Ingle.[1] When the animals reached 250 ± 2 g, they were anesthetized (intraperitoneal injection of 18 mg of cyclopentenyl-allyl-barbituric acid sodium) and subjected to the second stage of evisceration. Intravenous infusions of solutions containing glucose (C.P. Dextrose, Merck) and insulin (Regular Insulin, Lilly) were made by a continuous injection machine which delivered fluid into the saphenous vein of the right hind leg at the rate of 20 cc per 24 hours. The glucose load was 44 mg per 100 g of rat per hour and the insulin dose was 4 units per 24 hours per rat. Temperature was constant at 26.5 ± 0.5°C.

The time of survival was determined by use of a heart-beat amplifier (Model A, Upjohn) which was designed to amplify the D.C. potential generated by the heart beat to actuate a 6-point recording mechanism (Leeds and Northrop Micromax S). This apparatus gives a permanent visual record of an all-or-none response of the amplifier to the beating heart of 6 animals simultaneously.

Experiments and results. It is well known that the presence of inorganic ions other than sodium and chloride favor the survival of perfused isolated organs and tissues. In Experiment 1, a comparison was made of the survival times of eviscerate rats given Tyrode's solution with the survival times of similar rats given 0.9% sodium chloride solution. The amount of added glucose and insulin was the same in each group (see methods). Twenty-one rats given Tyrode's solution had an average survival of 2822 minutes with a range of 2457 to 3580 minutes. An equal number given saline had an average survival of 2818 minutes with a range of 2350 to 3262 minutes. It was concluded that either the use of Tyrode's solution failed to prolong survival to a greater extent than saline or that the effect was small.

As the eviscerate rat approaches death, there is a significant decrease in the concentration of proteins in the blood plasma and there is a concomitant increase in the free fluid of the body cavities (Ingle, Prestrud, and Nezamis, unpublished data). It was considered possible that the loss of fluid could be decreased and survival prolonged by the intravenous administration of a solution of colloids which might remain in the blood and sustain its osmotic activity. A 5% solution of degraded and purified gelatin containing 0.9% sodium chloride (Plazmoid, Upjohn) was used. Glucose and insulin were added to the solution. Fourteen eviscerate rats given gelatin had an average survival of 2509 minutes with a range of 1985 to 3046 minutes. Sixteen similar rats given saline with glucose and insulin had an average survival of 2748 minutes with a range of 2364 to 3076 minutes. The administration of gelatin did not reduce the rate at which fluid accumulated in the body cavities. It was concluded that this preparation of gelatin did not prolong the survival of the eviscerate rats.

Although the normal rat has a high natural resistance to the infection of wounds, the resistance of the eviscerate rat to infection is very low. A few hours following evisceration the peritoneum and even the blood becomes heavily charged with bacteria. We have never observed septicemia and peritonitis following other operations in the rat. The following organisms have been recovered from the peritoneal fluid of rats 24 hours following evisceration: Pseudomonas, hemolytic streptococci, *E. coli*, staphylococci, and *Proteus vulgaris*. Our attempts to apply sterile technic, although adequate for all of the many other operative procedures which we have carried out on the rat, have been only partially successful in the eviscerate rat. The sterilization of the skin of the rat with a germicidal agent (Mercresin, Upjohn), the use of sterile instruments, the use of sterile solutions for the infusions, etc., have greatly reduced the extent of bacterial growth per unit time, and the peritoneal fluid of an occasional animal has remained sterile, but uniform success has not been attained. That infection is an important factor in limiting the survival of these animals is indicated by the following results.

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[1] Ingle, D. J., *Exp. Med. and Surg.*, 1949, **7**, 34.

In Experiment 3, 26 rats which were eviscerated without asepsis had an average survival of 2743 ± 109 minutes, whereas 26 rats which were eviscerated under "aseptic" conditions had an average survival of 3113 ± 67 minutes. The standard deviation of the difference (370) was 129. The ratio between the difference and its standard deviation was 2.9, thereby meeting the usual requirements of statistical significance.

Discussion. Although a solution containing potassium, calcium, magnesium, phosphate and bicarbonate ions in addition to sodium and chloride is superior to saline alone for the perfusion of isolated tissues, the administration of Tyrode's solution to the eviscerate rat failed to have a significantly more favorable effect on survival than physiological saline. The blood and tissues of the eviscerate rat contain an optimal concentration and distribution of these ions at the time of operation and it is probable that no lack of these substances develops during its survival.

Hepatic insufficiency is characterized by the development of ascites. The extent to which the loss of fluid from the blood into the body cavities may be due to a decrease in plasma proteins is not known to us. The administration of gelatin did not inhibit the rapid accumulation of fluid in the body cavities, nor did it prolong survival. Other plasma substitutes should be tested in the eviscerate rat.

The readiness with which the eviscerate rat develops septicemia and its low resistance to infection of the peritoneal cavity in contrast to the high resistance of the non-eviscerate rat offer interesting problems. The future success of our efforts to prolong the survival of the eviscerate rat will depend in part upon the outcome of our attempts to completely prevent infection. Even the partial success of our present efforts has significantly prolonged the survival of these animals. The average survival of the "aseptic" group of Experiment 3 is longer than has been reported in the literature for any series of liverless mammals. The longest survival of any single animal of our series to date is 63 hours and 47 minutes.

Summary. Eviscerate male rats were given continuous intravenous infusions of solutions containing glucose and insulin. The times of survival were determined by a heart-beat recorder. The substitution of Tyrode's solution and of 5% gelatin for 0.9% sodium chloride in the infusion fluid failed to prolong survivals. The rats which were eviscerated without sterile technic developed septicemia and peritonitis. The attempts to maintain asepsis during the procedure of evisceration were not uniformly successful, but the average survival of 26 rats operated under "aseptic" conditions was 3113 ± 67 minutes as compared to 2743 ± 109 minutes for 26 rats operated without asepsis. The maximum survival time recorded for any animal was 63 hours and 47 minutes.

We wish to express our appreciation to Mary Ann Nook, Department of Bacteriology, The Upjohn Laboratories, who carried out the bacteriologic studies for these experiments.

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17216. Experimental Lathyrism in the White Rat and Mouse.[*]

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One of us (L.) has recently reported studies of the production of experimental lathyrism in the white rat.[1] Of the species studied, the most characteristic symptoms resulted from the ingestion of the seeds of *Lathyrus odoratus* (the flowering sweet pea) while the seeds of *Lathyrus sylvestris Wagneri* (flat pea) were most toxic, since death resulted within a few days and after the consumption of as little as 4 g of the legume. The toxic principle was readily extracted with 30% ethanol in every case.

In the present study, we report observations on the seeds of another species of *Lathyrus, Lathyrus latifolius* (the perennial or everlasting pea), commonly grown for its flowers in gardens.[†] The finely ground meal was incorporated into the diet of young male white rats (50-60 g) at the 50% level as in previous experiments.[1] Animals fed the *Lathyrus latifolius* meal diet showed signs of marked intoxication and died in from 3 to 8 days after the beginning of the experiment. A typical protocol in condensed form is presented here. A rat of 70.4 g gained 8 g and consumed 20 g of the diet (*i.e.*, 10 g of the legume) in the first 2 days of the experiment. On the 3rd day, 4.3 g were eaten and on the 3 following days, 0.4 to 1.0 g. Violent convulsions, marked reactions to external stimuli such as tapping the cage with a rod, and partial paralysis appeared on the 5th day and death resulted on the 6th day. A similar picture was presented in 6 other rats which received the *latifolius* meal. The symptoms were similar in most respects to those already reported in rats fed seeds of *Lathyrus sylvestris Wagneri*. Control rats fed the edible white pea of commerce (*Pisum sativum*, var. *arvense*) ate well, gained steadily and showed no signs of toxicity. It is evident that this species of *Lathyrus* was more toxic for rats than was any of the other species previously studied in this laboratory except *L. sylvestris Wagneri.* Mr. Roland McKee of the United States Department of Agriculture in a private communication received before the experiments with *Lathyrus latifolius* were undertaken, has stated that "I have always considered this species as very closely related to *L. sylvestris.*" It is of considerable interest that the toxicity of *latifolius* is so similar to that of *sylvestris Wagneri.*

Marked variations in the susceptibility of domestic or laboratory animals to *Lathyrus* intoxication have been observed.[2,3] We have studied in young white mice the effects of the consumption of the seeds of *Lathyrus odoratus,* the species of *Lathyrus* with which most typical symptoms of experimental lathyrism in the young white rat were observed. The diet was similar to that used in previous experiments with rats.[1] However, since the protein requirement of the diet of the smaller animal, the white mouse, is greater than that of the rat, the amount of casein was increased from 10 to 15%, the extra casein replacing an equivalent weight of starch. When young male white mice of 12-15 g weight were fed diets containing 50% of *odoratus* meal, they grew well until a weight of approximately 25-30 g was reached. The increments of weight were in every respect similar to those of pair-fed control mates which received diets containing *Pisum sativum*. The gains per 100

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[*] This work has been made possible by a grant from the Board of Governors of the Horace H. Rackham School of Graduate Studies of the University of Michigan.

[1] Lewis, H. B., Fajans, R. S., Esterer, M. B., Chen, C.-W., and Oliphant, M., *J. Nutrition,* 1948, **36**, 537.

[†] Obtained through the cooperation of Rex D. Pearce of Moorestown, N. J.

[2] Schuchardt, R., *Deut. Arch. klin. Med.,* 1886-87, **40**, 312.

[3] Stockman, R., *J. Pharm. Exp. Therap.,* 1929, **37**, 43.

g of food consumed for the first 20 days were essentially the same, 11.8 and 11.0, respectively. Although the diet was maintained over a period of 17 weeks, no symptoms of lathyrism were observed. Since we were using a new lot of *L. odoratus* peas, we felt it necessary to confirm previous observations of the toxicity of this species for rats. Accordingly, we repeated our previous experiments[1] with the new shipment of sweet pea seed. All the rats developed characteristic experimental lathyrism in from 3 to 6 weeks. In view of the failure of young white mice to exhibit any symptoms of lathyrism on diets containing *odoratus,* it is notable that the mice ate from 71 to 86 g (average 77 g) of diet per 100 g of body weight over a typical 4-day period (17th to the 20th day of the experiment). This consumption was twice that of the young white rats under similar conditions (37.5 g), rats which without exception showed signs of acute lathyrism.

Since white mice fed *odoratus* showed no evidence of toxicity, it was of interest to study the effects of diets containing the 2 species of *Lathyrus,* which had proven to be most injurious to rats, *i.e., Lathyrus sylvestris Wagneri* and *Lathyrus latifolius.* Three mice received diets containing 50% *latifolius.* None showed the marked irritability, convulsions or paralysis seen in rats fed this diet, but all lost weight rapidly, decreased the food consumption and died in 5, 10 and 12 days, respectively. Two mice, fed diets containing *latifolius* extracted with 30% ethanol as previously described[1] maintained their weight, ate the diet well and survived with no signs of toxicity for 18 days, at which time lack of available legume resulted in the termination of the experiment.

Three mice fed diets containing 50% of *L. sylvestris Wagneri* seed lost weight rapidly and died in 4, 6, and 7 days, respectively. Increased irritability and convulsions were observed, but these symptoms were, perhaps, slightly less acute than in young white rats.

It is of some interest to note that Schuchardt[2] in his classical paper on lathyrism states that *Lathyrus sylvestris* and *Lathyrus latifolius* afford "essbare Samen!"

Summary. 1. Young white mice of 12 to 15 g body weight fed diets containing 50% *Lathyrus odoratus* meal grew well and showed no development of experimental lathyrism in 17 weeks, although young white rats which received a similar diet developed experimental lathyrism in from 3 to 6 weeks. 2. Diets containing *Lathyrus latifolius* meal (50%) were toxic for both rats and mice, death occurring in from 3 to 8 days (rats) and 5 to 12 days (mice). The extraction of seed of *Lathyrus latifolius* meal with 30% ethanol removed the toxic principle. Mice fed the extracted meal survived and appeared normal over a period of 18 days. 3. Mice fed *Lathyrus sylvestris* seed died with symptoms of acute toxicity in 4 to 7 days.

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17217. Glycogen in Rachitic Cartilage and Its Relation to Healing.*

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The presence of glycogen in cartilage was described almost 100 years ago by Charles Rouget.[1] During the intervening years the feeling has grown that glycogen might have some relation to the calcification process. Marchand[2] noted the high glycogen content of hypertrophic cartilage cells in the region of lime salt deposition. Hoffmann *et al.*[3] observed that "a striking relationship exists between the disappearance of glycogen in car-

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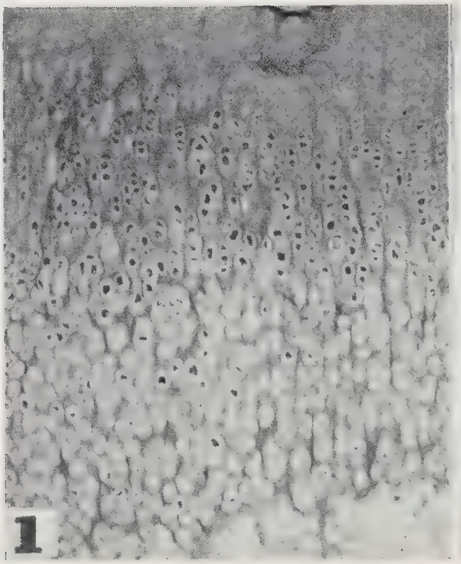
* Aided by a grant from Mead Johnson and Company.

[1] Rouget, C., *J. de la Physiol.*, 1859, **2**, 308.

[2] Marchand, F., *Virch. Arch.*, 1885, **100**, 42.

[3] Hoffmann, A., Lehmann, G., and Wertheimer, E., *Pfluger's Archiv.*, 1928, **220**, 183.



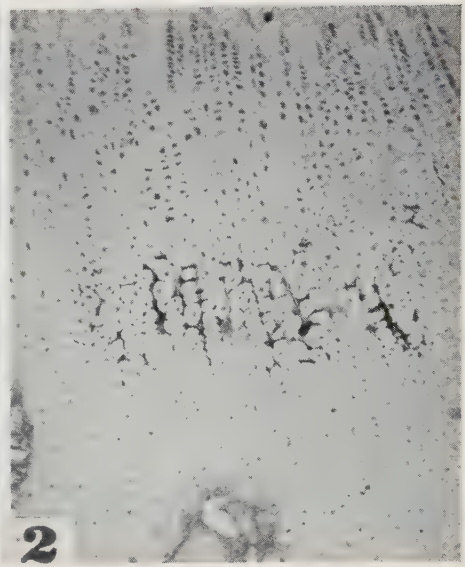


FIG. 1.

Epiphyseal cartilage of rachitic rat. Note widened zone of hypertrophic cells, the uppermost ones of which contain dark accumulations of glycogen. The cell nuclei stain poorly and should not be confused with the glycogen. Periodic acid-leucofuchsin technic.

FIG. 2.

Epiphyseal cartilage of rachitic rat (same magnification as Fig. 1) which had been given phosphate intraperitoneally 24 hours before. Note initial deposition of inorganic material in upper portion of hypertrophic cartilage corresponding to zone in which glycogen is found. Von Kossa technic.

tilage and its ossification." Harris,[4] shortly thereafter, suggested that glycogen might have a bearing on the process of calcification, possibly being related to Robison's[5] phosphatase mechanism. More recently, the presence of glycogen in hypertrophic cartilage cells has assumed further importance as a result of Gutman's[6] demonstration of phosphorylase activity in epiphyseal cartilage.

The following observations, which are part of a general histochemical survey of normal and diseased cartilage and bone,[7] are briefly reported because of the further evidence they bring to bear on the relationship of glycogen to the calcification mechanism. Weanling rats were placed on the Steenbock rachitogenic diet (No. 2965) supplemented with crystalline vitamins. After one to 5 weeks on this regimen some of the rats were injected intraperitoneally with 2.5 cc per 100 g of animal of a mixture of 1 part M/10 NaH_2PO_4 and 4 parts M/10 Na_2HPO_4 to produce healing as recommended by Urist and McLean.[8] Such animals were killed from 12 to 24 hours afterward; uninjected rachitic controls were likewise sacrificed. The upper end of the tibia was fixed in neutral formol-alcohol and, after sectioning without decalcification, stained for glycogen by the Bauer or McManus technics and for inorganic materials by the von Kossa procedure.

Glycogen is not present in the widened zone of hypertrophic cells, so characteristic of rickets. Glycogen is present in the usual increasing amounts in the cells which are undergoing the normal maturation cycle at the upper margin of the widened zone of hypertrophic cells. In other words, glycogen appears in normal fashion as the cartilage cell reaches its full stage of differentiation; as the cell persists, however, for reasons which are not clear at this time, the glycogen disappears. In the rachitic animals in which healing had not been induced, no deposition of inorganic material is present in the vicinity of the hypertrophic glycogen containing cells, or those from which glycogen has disappeared. When healing was instituted, it took place, as has often been noted both *in vivo*[9] and *in vitro*,[10] in the matrix adjacent to those cartilage cells which have most recently matured, that is, in the immediate vicinity of the cells which contain abundant glycogen and, in the early period of observation, not down farther in the matrix adjacent to the hypertrophic cells devoid of glycogen. There is thus a regional relationship between the presence of glycogen and deposition of inorganic elements as demonstrated by the von Kossa technic. It is, of course, impossible to determine any precise reciprocal relationship between the disappearance of glycogen and the appearance of inorganic materials since to follow the disappearance of one and the appearance of the other is not possible. It is hoped that quantitative methods[11] now available, using larger animals, can be applied to study this particular problem.

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[4] Harris, H. A., *Nature*, 1932, **130**, 996.

[5] Robison, R., *Biochem. J.*, 1923, **17**, 286.

[6] Gutman, A. B., and Gutman, E. B., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 687.

[7] Follis, R. H., Jr., and Berthrong, M., *Am. J. Path.*, 1948, **24**, 685.

[8] Urist, M. R., and McLean, F. C., *J. Bone and Joint Surg.*, 1941, **23**, 283.

The glycogen content of rachitic cartilage has received little attention. Some years ago Suppes[12] reported observations on postmortem human material and concluded that rachitic cartilage did not contain glycogen. No mention was made of the presence of glycogen in the uppermost hypertrophic cells so that one suspects autolytic changes may have been a complicating factor.

The localization of glycogen in rachitic cartilage in the region where calcification first appears during healing is, of course, important in any theory of calcification which has the glycogenolytic cycle as its basis.[13] More precise localization of phosphorylase activity[6] as well as that of other enzymes which participate in the cycle will have to be carried out before such an hypothesis can be fully accepted.

Summary. Glycogen is present in rachitic cartilage only in the most recently matured hypertrophic cells. The broad zone of hypertrophic cells beneath is devoid of glycogen. This localization coincides with the area where inorganic elements deposit during the initial stages of healing and furnishes further evidence for the relationship of glycogen, and possibly the glycogenolytic cycle, to the calcification mechanism.

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[9] McCollum, E. V., Simmonds, N., Shipley, P. G., and Park, E. A., *J. Biol. Chem.*, 1922, **51**, 41.

[10] Shipley, P. G., Kramer, B., and Howland, J., *Biochem. J.*, 1926, **20**, 379.

[11] Follis, R. H., Jr., *Fed. Proc.*, 1949, **8**, 480.

[12] Suppes, J., *Frank. Z. Path.*, 1922, **26**, 268.

[13] Gutman, A. B., Trans. Conf. on Metabolic Aspects of Convalescence, Josiah Macy, Jr. Foundation, 1946, **14**, 20.

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17218. Simultaneous Administration of Adrenal Cortical Extract and Desoxycorticosterone; Effects on Blood Pressure of Hypertensive Patients.*

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Previous studies[1] have indicated that the continued administration of an adrenal cortical extract may be associated with a decrease in the "resting" blood pressure of patients with

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* This investigation was supported, in part, by a research grant from the National Heart Institute, U. S. Public Health Service, and was aided through the generosity of the Albert and Mary Lasker Foundation.

[1] Pines, K. L., Perera, G. A., Vislocky, K., and Barrows, A. D., Proc. Soc. Exp. Biol. and Med., 1948, **68**, 286.

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uncomplicated hypertensive vascular disease. Inasmuch as the daily injection of desoxycorticosterone acetate has consistently produced at least a transitory and sometimes a sustained pressor response under comparable conditions,[2,3] the effect of both agents was observed when given in combination.

Methods. Two men and 3 women with uncomplicated hypertensive vascular disease were studied on the metabolism ward of the Presbyterian Hospital. The criteria in selection of patients and the methods employed were identical to those previously described.[4] All subjects were maintained on a constant dietary, fluid and sodium chloride intake. Following a 3-week baseline period, desoxycorticosterone acetate† (DCA) was administered subcutaneously in 5 mg doses twice daily, and adrenal cortical extract (Upjohn) (ACE) was given intramuscularly in 10 cc doses twice daily, both materials being injected for

TABLE I.
Laboratory Data of 5 Hypertensive Patients Before and During Treatment with DCA and ACE.

Wt, k	Urine*				Serum				
	Vol. cc	Sodium	Chloride	Hematocrit % cells	Urea N, mg/100 cc	Proteins, g/100 cc	Na	Cl	K
		milliequiv.						milliequivs/l	
A† P†				A P	A P	A P	A P	A P	A P
65.5–66.0	1840–1580	96–64	85– 50	40.3–37.3	17–15	6.2–6.1	140.0–142.3	99.6–102.0	3.8–3.3
65.6–66.3	1400–1400	57–42	69– 58	46.0–46.0	10– 8	6.4–6.4	139.3–138.3	102.2–100.2	4.7–4.2
78.5–78.4	1460–1520	74–56	94– 75	—	—	—	136.3–137.4		4.4 4.3
63.2–64.0	1540–1400	84–60	98– 72	45.0–44.1	12–10	6.6–6.5	138.9–138.6	99.2–100.0	3.9 3.1
65.0–65.6	1620–1480	—	118–100	—	—	7.5–7.4	138.6–138.7	—	5.2 5.2

* Mean daily values for week preceding and week of steroid administration.
† A = Start of treatment.
P = End of treatment.

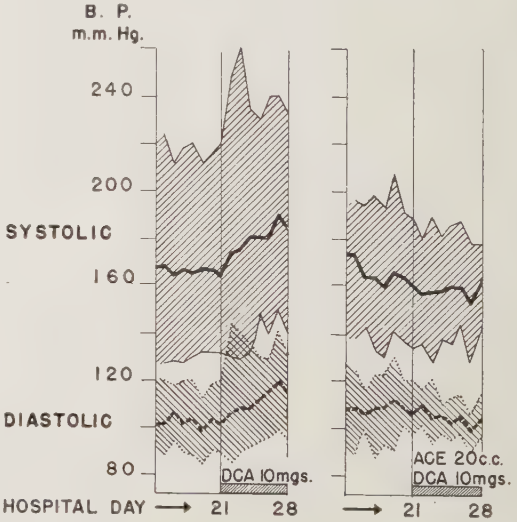

FIG. 1.

Maximum, minimum and mean systolic and diastolic blood pressures of 12 hypertensive patients given DCA and 5 given both DCA and ACE.

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[2] Perera, G. A., and Blood, D. W., *Ann. Int. Med.*, 1947, **27**, 401.

[3] Perera, G. A., PROC. SOC. EXP. BIOL. AND MED. 1948, **68**, 48.

[4] Perera, G. A., and Blood, D. W., *J. Clin. Invest.*, 1947, **26**, 1109.

† Desoxycorticosterone acetate (DOCA) was supplied through the generosity of Dr. K. W. Thompson of Roche-Organon, Inc., Nutley, N. J.

a period of a week. The results were compared to those previously obtained in 14 patients receiving only DCA,[2] two of whom being included in the present series after repeatedly demonstrating a prompt rise in blood pressure in association with this steroid given alone.

Results. The combination of DCA and ACE generally produced increases in weight, hemodilution, reductions in urinary volume, and sodium and chloride retention. These changes were similar to those observed after DCA alone. No consistent alterations in serum sodium or chloride values were noted, but reductions in serum potassium levels were again evident in the majority (Table I). In no instance was fever, leucocytosis, eosinophilia or rise in erythrocyte sedimentation rate produced by these agents. In all patients there were either minimal changes in "resting" blood pressure or an actual decline (Fig. 1).

Conclusions. In patients with hypertensive vascular disease, the simultaneous administration of an adrenal cortical extract appears to block the pressor effect of desoxycorticosterone acetate when used alone.

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17219. Effect of 11-Desoxycorticosterone Acetate upon Carbohydrate Utilization by the Depancreatized Rat.*

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In recent years it generally has been thought that only those steroids of the adrenal cortex which have an oxygen atom at the 11 position in ring C can directly influence carbohydrate metabolism. In particular, the synthetic compound 11-desoxycorticosterone, which has not even been demonstrated with certainty to occur in the gland and which is exceedingly potent in causing sodium retention, has been considered to have no carbohydrate action. However, Verzar and his co-workers have demonstrated[1] that this steroid may reduce glycogen synthesis in isolated diaphragm muscle and may prevent the increased glycogen formation produced in this tissue by insulin. Using glucose labeled with carbon 14, this observation has been confirmed and extended in this laboratory.[2]

Ingle[3] recently has found that in rats made diabetic by partial pancreatectomy, 11-desoxycorticosterone acetate caused a slight decrease in the level of urinary glucose when given in small doses, while large doses of the steroid led to a marked exacerbation of the glycosuria.

Our experiments were carried out in an attempt to find out if desoxycorticosterone would oppose the action of insulin in the diabetic organism.

Methods. Male albino rats of the Slonaker strain[4] were depancreatized at 6 weeks of age in the usual manner.[5] The rats were maintained on Purina Chow until 1 week before the start of the experiment at which time they were fed the following rather high carbohydrate diet: Sucrose 50, casein 18, yeast 10, salt mixture 4, C.L.O. 4, lard 14. After their diabetes was stabilized on this above diet, the animals were placed in individual metabolism cages. The rats were weighed and their food intake determined daily. The urine was

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* This investigation was supported by a research grant from the Division of Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service.

[1] Verzar, F., and Wenner, V., *Biochem. J.*, 1948, **42**, 35; 1948, **42**, 48.

[2] Bartlett, G. R., Wick, A. N., and MacKay, E. M., *J. Biol. Chem.*, 1949, **178**, 1003.

[3] Ingle, Dwight J., Proc. Soc. Exp. Biol. and Med., 1948, **69**, 329.

[4] MacKay, L. L., *Am. J. Physiol.*, 1928, **86**, 215.

[5] Pauls, Frances, and Drury, Douglas R., *J. Biol. Chem.*, 1942, **145**, 481.

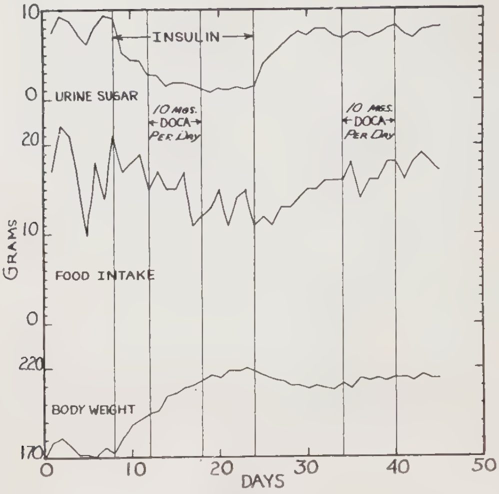

Fig. 1.

Effect of insulin and DOCA on food intake. urine sugar and body weight in depancreatized rats.

collected with a preservative in 24 hour samples and sugar excretion determined by the Shaffer-Somogyi method.[6] Protamine zinc insulin[†] was administered in doses of 1 unit 2 times daily. Desoxycorticosterone acetate (DOCA)[‡] was made up in oil at a concentration of 10 mg per ml. It was given by subcutaneous injection in divided doses each morning and late afternoon. Food was given *ad libitum*.

Results. Fig. 1 presents the significant data as averages for 6 rats. The figures for the individual rats show no important differences from the averages. As usual insulin decreased the food intake and increased the weight gain by reducing the glycosuria. The desoxycorticosterone acetate in a dose of 10 mg per rat per day for 6 days had no influence upon the action of insulin. The DOCA also had no influence when given in the same dose without insulin during an intense diabetes.

Discussion. At first glance our results appear to be at variance with those reported by Ingle.[3] This investigator's rats had a milder diabetes than ours and were force fed with a constant food intake. In our experiments the food intake varied with the desire of the animals. Had the food intake been fixed at the high pre-insulin level during the period of insulin administration, the DOCA administration might have opposed the insulin action, which was largely one of storing sugar.[5] It does seem odd that when no insulin was being given, DOCA in large doses failed to affect the glycosuria. This difference from Ingle's results may be due to the greater severity of the diabetes in our rats which may have been so severe that an exacerbation was not possible.

Summary. Severely diabetic depancreatized male rats reacted as usual to protamine zinc insulin administration by reducing their food intake but gaining weight through a reduction in the glycosuria. Desoxycorticosterone acetate in large doses was without measurable influence on the body weight gain or glycosuria. The glycosuria of the severely diabetic rat without insulin therapy and receiving food *ad libitum* was unaffected by similar doses of desoxycorticosterone acetate.

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[6] Shaffer, P. A., and Somogyi, M., *J. Biol. Chem.*, 1933, **100**, 695.

[†] We are indebted to Dr. F. B. Peck of the Lilly Research Laboratories for a supply of insulin.

[‡] Dr. Edward Henderson of the Schering Corporation supplied the desoxycorticosterone acetate ("Cortate") in oil which was used.

17220. Effect of Folic Acid, "4-Amino" Folic Acids and Related Substances on Growth of Chick Embryo.*

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(Introduced by C. P. Rhoads.)
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Pteroylglutamic acid (folic acid) has an essential role in the growth of bacteria, protozoa, insects, birds and mammals.[1] Several weak folic acid antagonists were first described in 1947,[2,3] and shortly afterwards Seeger *et al.*[4] discovered 4-amino pteroylglutamic acid, an extremely powerful antagonist to folic acid by the *Streptococcus fecalis* R test. Franklin *et al.*[5] found that 4-amino PGA was toxic to mice, and that folic acid offered only slight protection, and this within a narrow dose range. 4-amino PGA and some of its related componnds produced a toxic picture in mammals somewhat similar to that of other systemic cell poisons, such as x-rays and nitrogen mustard, with lymph node regression, leucopenia and thrombocytopenia with bone marrow aplasia, diarrhea with sloughing of the intestinal mucosa, and death within 3 to 4 days after a single acute dose. Philips, Thiersch *et al.*[6,7,8] have reviewed the pharmacology of these compounds and they conclude that the "4-amino" compounds are true folic acid antagonists, but are unique as anti-metabolites in that they induce an acute and absolute folic acid deficiency physiologically, even in the presence of folic acid. Because of the actions of the "4-amino" folic acids, a study was made of the effect of these and related compounds on the growth of the chick embryo.

Methods. Fertile White Leghorn eggs were obtained from a commercial source. The eggs were incubated at 100°F, and a relative humidity of 75%. In most instances the embryos were injected with the test agent at 4 days of incubation. The chemicals were dissolved in sterile saline and injected into the yolk sac by introducing a No. 25 needle through a small hole at the blunt end of the egg. The total volume injected into each egg ranged between 0.1 and 0.2 cc; larger volumes were sometimes necessary for the large doses of the relatively non-toxic drugs. When most of the embryos in a given group were dead, those remaining were sometimes sacrificed for detailed examination. The toxicity experiments were usually concluded by sacrificing the embryos 8 to 10 days after injection, when the embryos were 12 to 14 days of age.

The 15 compounds used in this study are listed in Table I.

Results. After considerable exploration, the 4 day old embryo was selected as most

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* This study was aided by a grant from the Jane Coffin Childs Memorial Fund for Medical Research and the National Cancer Institute of the National Institute of Health, United States Public Health Service. Most of the drugs used were supplied by the Lederle Laboratories Division and the Calco Laboratories of the American Cyanamid Company; 2,6-diaminopurine was supplied by the Wellcome Research Laboratories.

[1] Jukes, T. H., and Stokstad, E. L. R., *Physiol. Rev.*, 1948, **28**, 51.

[2] Franklin, A. L., Stokstad, E. L. R., and Jukes, T. H., Proc. Soc. Exp. Biol. and Med., 1947, **65**, 368.

[3] Hutchings, B. L., Mowat, J. H., Oleson, J. J., Stokstad, E. L. R., Boothe, J. H., Waller, C. W., Angier, R. B., Semb, J., and SubbaRow, Y., *J. Biol. Chem.*, 1947, **170**, 323.

[4] Seeger, O. R., Smith, J. M., Jr., and Hultquist, M. E., *J. Am. Chem. Soc.*, 1947, **69**, 2567.

[5] Franklin, A. L., Stokstad, E. L. R., and Jukes, T. H., Proc. Soc. Exp. Biol. and Med., 1948, **67**, 398.

[6] Philips, F. S., and Thiersch, J. B., *J. Pharm. and Exp. Therap.*, 1949, **95**, 303.

[7] Philips, F. S., Thiersch, J. B., and Ferguson, F. C., *Ann. New York Acad. Sci.*, in press.

[8] Thiersch, J. B., and Philips, F. S., *Am. J. Med. Sci.*, 1949, **217**, 575.

[9] Hitchings, G. H., Elion, G. B., VanderWerff, H., and Fako, E. A., *J. Biol. Chem.*, 1948, **174**, 765.

TABLE I. Toxicity of Folic Acid and Allied Compounds to the 4 Day Old Chick Embryo.

Compound	Dose mg	No. embryos	Embryo deaths, or sacrifices () or¹ age embryo, days										Approx. LD $_{50}$, mg/egg	Abnormality incidence in embryos surviving beyond 9 days of age
			5	6	7	8	9	10	11	12	13	14		
I. Pteroyl glutamic acid	20	15	0	0	2	1	0	0	0	0	(9)	(3)	20	0/12
II. ,, diglutamic acid	20	5	0	0	0	0	1	0	0	0	(4)	—	20	0/4
III. ,, triglutamic ,,	20	8	0	0	0	1	0	1*	0	0	(6)	—	20	1/7*
IV. 4-amino pteroylglutamic	.008-.010	29	0	14	10	2	(1)	1^1	—	—	—	—		2/2
acid	.004-.006	37	0	5	7	2	5^1	4	5^1	1^3	0	(3)	.003	8/17
	.002-.003	16	0	0	0	0	2	4	2	1	1^5	(1)		7/14
V. 4-amino N¹⁰methyl	.009-.013	16	1	7	0	1	2	(2)	0	2	1	—		5/5
pteroylglutamic acid	.005-.006	16	2	3	1	0	2	1	0	(1)	(3)	1^2	.005	1/8
	.003	6	0	0	0	0	0	0	0	0	(6)	—		1/6
VI. 4-amino 9 methyl	.010	11	0	4	4	1	0	0	0	0	(2)	—		0/2
pteroylglutamic acid	.004-.005	17	1	3	3	3	2	2	1	1	(1)	—	.003	2/5
	.001-.003	21	2	2	2	2	0	0	1	1	1^9	(1)		2/13
VII. 4-amino 9-10 dimethyl	.004-.005	23	0	4	9	2	4	1^1	0	(2)	—	—		3/4
pteroylglutamic acid	.002-.003	20	1	1	1	1	2	2	2	1^7	1	(1)	.003	4/14
VIII. 4-amino pteroylaspartic	.400-.600	15	2	9	0	2	2	—	—	—	—	—		
acid	.150-.250	15	0	6	4	3	1	0	(1)	—	—	—	.075	1/1
	.050-.100	26	1	5	7	1	3	0	0	0	(5)	(4)		7/9
	.025	9	0	0	0	0	0	0	0	0	0	(9)		0/9
IX. 4-amino pteroylthreonine	4-5	8	0	1	2	4	0	1	—	—	—	—		1/1
	2	9	0	2	0	2	0	0	1	2^2	—	—	1.5	5/5
	0.5-1	10	2	0	0	0	0	0	0	1	2	(5)		1/8
X. 4-amino pteroylalanine	2	5	1	0	2	2	—	—	—	—	—	—		
	1	5	0	1	1	0	0	0	0	0	0	(3)	1.5	1/3
	0.5	5	0	0	1	0	1	0	0	0	0	(3)		0/3
XI. N¹⁰methyl pteroic acid	5-6	21	6	8	1	2	0	0	(1)	0	(3)	—		0/4
	4	5	1	1	0	0	0	0	0	0	0	(3)	4	0/3
	2	15	2	2	0	1	0	0	(2)	(4)	(4)	—		0/10
XII. Pteroylaspartic acid	20	6	1	0	0	0	0	1	0	0	(4)	—	20	0/5
(dextro)	10	6	0	0	0	0	0	0	0	0	(6)	—		0/6
XIII. Pteroylaspartic acid	20	5	0	0	1	0	0	0	0	0	(4)	—	20	0/4
(racemic)	10	6	0	0	0	1	0	0	0	0	(5)	—		0/5
XIV. 9-methyl pteroylglutamic	10	17	2	5	1	0	2	1	0	(1)	(3)	(2)	10	0/7
acid	4-6	43	8	11	0	4	2	0	0	1^1	(16)	—		0/18
	2	12	0	0	0	1	1	0	(1)	0	1^8	—		0/10
XV. 2,6-diaminopurine	4-5	41	6	5^1	5	3	8	6^7	—	—	—	—		See
	2-3	27	2	1	2	1	6^3	1	2^2	2^5	—	—	1	description
	1-1.5	15	1	0	0	1	0	1	0	2	2	4^4		below
	.25-.50	6	0	0	0	0	1	0	0	2	1	(2)		(in text)

* A stunted, anophthalmic embryo, not typical of "4-amino" PGA; this probably represents a spontaneous abnormality.

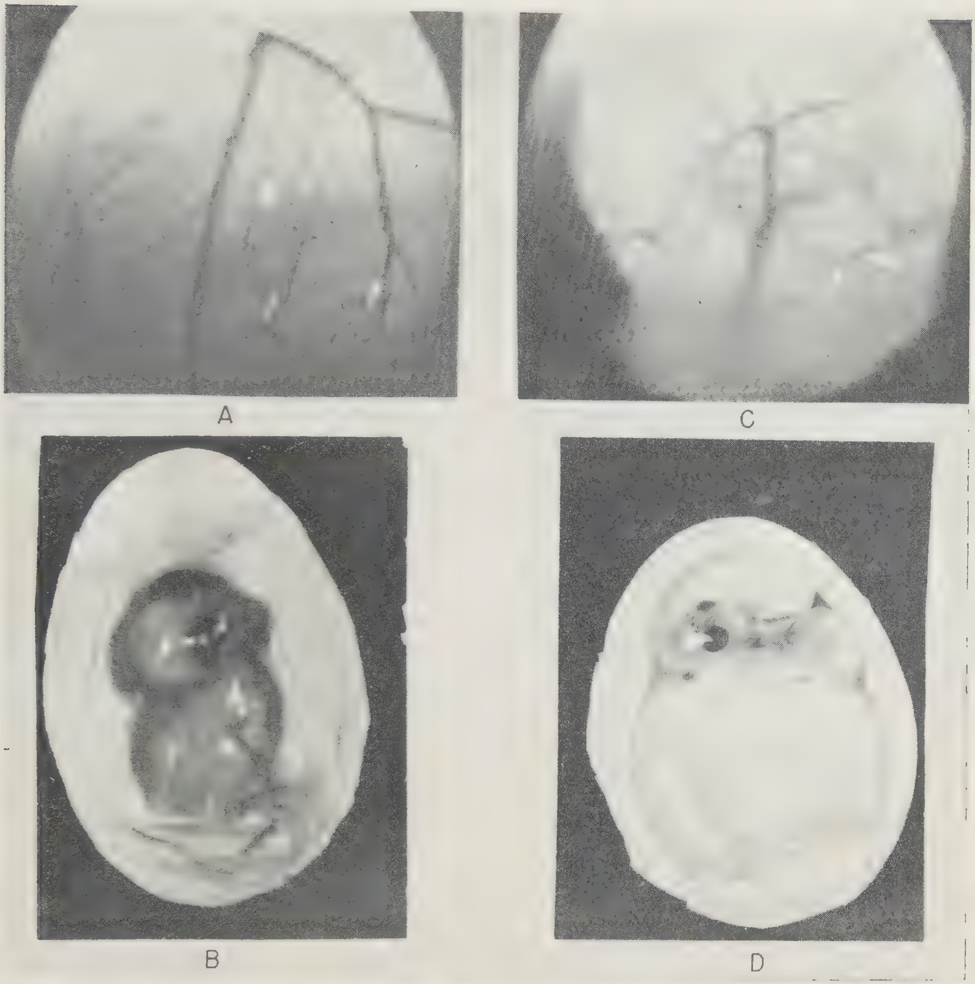

Fig. 1.

Appearance of a normal and stunted embryo, on candling, and in the opened egg. The normal appearing embryo received 0.003 mg/egg of 4-amino 9-methyl PGA at 4 days of age, sacrificed at 11 days of age. A) The vascular pattern, as seen by candling, appears normal. B) The embryo appears to be normally developed, and the allantois has extended laterally to envelop the contents of the egg. The abnormal embryo received 0.004 mg/egg of 4-amino 9-methyl PGA at 4 days of age, sacrificed at 11 days of age. C) The vascular pattern of the allantois, as seen by candling, is limited to a small area, about 3 × 3 cm, and it is separated from the yolk vessels. D) The embryo is severely stunted and abnormal, the amniotic sac is relatively enlarged, and the allantois is a large sac full of clear liquid.

satisfactory for routine use. Viability was easily determined by candling. The 4 day old embryos seemed to survive toxic doses of the "4-amino" folic acids for longer periods than the younger embryos, and developmental abnormalities were more conspicuous in those embryos surviving for longer periods. Older embryos required larger doses of the drugs, and developmental abnormalities were not as apparent.

Toxicity determinations in the chick embryo are subject to considerable variability, which may be attributed to some extent to differences in the genetic and nutritional background of the embryo, the season of the year, the age of the embryo at treatment, and the trauma associated with the injection. The LD_{50} figures are, therefore, approximate and represent the minimum dose range in which the compound shows activity. The

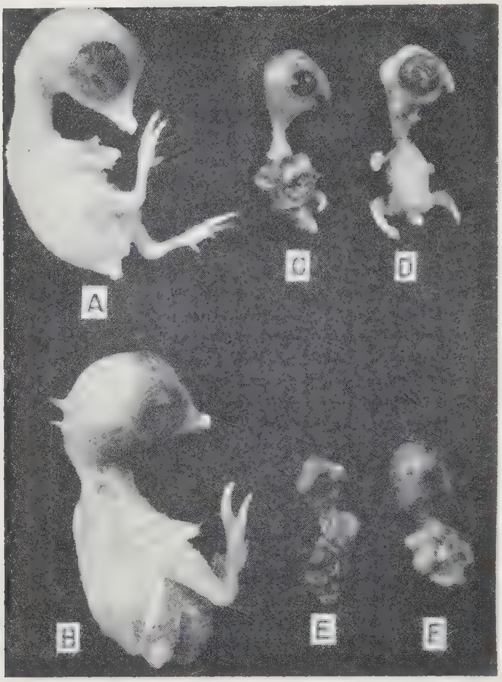

Fig. 2.

Appearance of normal and "4-amino" folic acid treated embryos. These were treated at 4 days of age and sacrificed 8 to 9 days later.

A. 12 day, control.

B. 13 day, control.

C. 2 mg 4-amino pteroylthreonine, sacrificed at 12 days.

D. 0.005 mg 4-amino 9-10 dimethyl PGA, sacrificed at 12 days.

E. 0.010 mg 4-amino N^{10} methyl PGA, sacrificed at 13 days.

F. 0.100 mg 4-amino pteroylaspartic acid, sacrificed at 13 days.

toxicity data for the compounds tested are summarized in Table I

Folic acid and its conjugates, pteroyl mono-(I), di-(II) and tri-(III) glutamic acids do not have an appreciable toxicity in the chick embryo at doses up to 20 mg/egg.

The "4-amino" folic acids produce a uniform toxic effect on the chick embryo, but alterations in the "4-amino" anti-folic molecule may affect the LD_{50} dosage. The LD_{50} of 4-amino PGA (IV) is 0.003 mg/egg; this LD_{50} dose is not appreciably altered by placing a methyl group in the N^{10} position (V) and in the 9 position (VI), and in the 9 and 10 position (VII). Substituting aspartic acid (VIII), threonine (IX) and alanine (X) for glutamic acid, however, increases the LD_{50} to approximately 0.075, 1.5 and 1.5 mg/egg, respectively.

The abnormalities produced in the embryo by the "4-amino" compounds have been remarkable and fairly uniform. The embryos dying during the first 4 days after treatment are severely stunted, but they are usually too autolyzed to permit a useful examination. Those embryos surviving for 8 to 10 days show the most remarkable changes. The appearances of the allantoic vessels in an 11 day normal and abnormal embryo are shown in Fig. 1, A,C. On opening these eggs the reason for the vascular abnormality is apparent. (Fig. 1, B, D). The normal embryo weighed about 4 g and the chorioallantoic membrane extended widely. The treated embryo was severely stunted, weighing about 0.8 g. The amnion was distended with clear fluid. The allantois remained as a large fluid-filled sac, which was in connection with the shell over a relatively small area, and its blood vessel network was appropriately restricted.

Several abnormal embryos and controls of the same age are shown in Fig. 2. These embryos were all sacrificed. Whereas at 12 to 13 days of age the chick embryo normally weighs from 5 to 7 g, the treated embryos weighed from 0.8 to 1.2 g; the weight of a 7 day embryo. Besides the profound stunting, the abnormalities usually present were a flattened head, small eyes which frequently had a clear sac over the lens suggesting an enlarged anterior chamber, absent or underdeveloped lower beak, a long and usually twisted neck, exteriorization of the viscera, a fluid-filled sac surrounding the heart, abnormal or absent toes and various degrees of generalized edema. The abdominal organs appeared to be growing relatively more rapidly than the rest of the embryo. Occasionally, intermediate stages were seen, with larger embryos showing generalized edema, abnormal beaks, absence of feathers and limb abnormalities. Usually, however, those embryos surviving 8 to 10 days after treatment showed either the typical stunting shown in Fig. 2 or they were apparently normal. The oldest stunted embryo we have seen was 14 days old; it is unlikely that many can survive beyond this age.

These embryo abnormalities were not produced by pteroyl aspartic acid (dextro) (XII) and (racemic) (XIII), by 9 methyl pteroylglutamic acid (XIV), also weak folic acid antagonists[2,3] and by N^{10} methylpteroic acid (XI).

2,6-diaminopurine is included in this report because Hitchings *et al.*[9] found that it acted as an antagonist of folic acid over a limited range, and as an antagonist of adenine over a more extended range, in the growth of *Lactobacillus casei.* It also damages the hematopoietic system of laboratory animals,[10] and prolongs the survival time of mice with transplanted leukemia,[11] actions qualitatively similar to those of the "4-amino" anti-folics.[6,12]

In the 4-day-old chick embryo, 2,6-diaminopurine is a moderately toxic compound, the LD_{50} being in the range of 1 mg/egg. The mortality pattern seems to differ appreciably from the "4-amino" compounds in that, in the LD_{50} range, many of the embryos may survive for longer than 5 days. At this dose, the embryos surviving for longer than 5 days did not appear appreciably stunted, but they were extraordinarily pale and a few random hemoglobin determinations showed values much lower than the normal. Probably as a result of the anemia, cardiac enlargement was also conspicuous in some of these embryos. Other findings were less constant, and they included generalized edema, abnormal lower beaks, and necrotic areas in the liver. Daniel *et al.*[13] found that 2-amino-4-hydroxy-6,7-dimethyl pteridine and 2-amino-4-hydroxy-6,7-diphenylpteridine inhibited growth and hemoglobin formation in the chick, but the effect could be counteracted by folic acid. The toxicity of 2,6-diaminopurine in the egg, however, could not be altered by adenine (20 mg/egg) or by folic acid (10 mg/egg).

Attempts to determine whether folic acid provided the chick embryo with some protection against the "4-amino" compounds have been unsuccessful. Twenty mg of folic acid were given almost simultaneously with different dose levels (multiples of the approximate LD_{50}) of 4-amino PGA (IV) and 4-amino N^{10} methyl PGA (V), a weight ratio of 1000 to 4000 to 1, but no clear-cut protection could be demonstrated. There were several experiments in which folic acid protected the chick embryo against 4-amino pteroyl aspartic acid, but this could not be demonstrated consistently. There is no evidence in these experiments that folic acid, within the closed system provided by the egg, gave any significant protection against the "anti-folic" compounds; in fact, the stunted embryos, produced by the "4-amino" anti-folics, occurred in the presence of folic acid.

Discussion. It is not clear whether the profound stunting of the chick embryo produced by the "4-amino" anti-folics is due to a general interference with the growth of the embryo or due to injury to a specific tissue which leads to retardation in the growth of the embryo. Wagley and Morgan[14] found that the "4-amino" PGA's in the chick embryo produced marked cytological changes in the yolk sac blood islets, and we have observed that the vascular network of the chorioallantoic membrane is considerably reduced in size in the stunted embryos. Hertz and Tullner,[15] in demonstrating that "4-amino" PGA inhibits the response of the chick oviduct to estrogen stimulation, also implied that it acted on specific tissues. We tend to favor the more general interpretation, however, that the "4-amino" folic acids interfere with the growth of tissues—those growing more rapidly being most obviously affected—and the thesis that it interferes with the growth of certain tissues, in a highly specific fashion, remains to be proved.

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[10] Philips, F. S., and Thierch, J. B., *Fed. Am. Soc. Exp. Biol., Proc.*, 1949, **8**, 325.

[11] Burchenal, J. H., Bendich, A., Brown, G. B., Elion, G. B., Hitchings, G. H., Rhoads, C. P., and Stock, C. C., *Cancer*, 1949, **2**, 119.

[12] Burchenal, J. H., Burchenal, J. R., Kushida, M., Johnston, S., and Williams, B. S., *Cancer*, 1949, **2**, 113.

[13] Daniel, L. J., Scott, M. L., Norris, L. C., and Heuser, G. F., *J. Biol. Chem.*, 1949, **173**, 123.

[14] Wagley, P. F., and Morgan, H. R., *Bull. Johns Hopkins Hosp.*, 1948, **83**, 275.

[15] Hertz, R., and Tullner, W. W., *Endocrinology*, 1949, **44**, 278.

The inability of folic acid to protect the chick embryo from the toxic action of the "4-amino" PGA's, even when given in ratios of 1000 to 4000 to 1, is further evidence of the difficulty in protecting against or reversing the activity of these compounds. Franklin *et al.*[5] have obtained slight and limited protection against "4-amino" PGA with large doses of folic acid, and Hertz and Tullner,[15] using a ratio of folic acid to 4-amino pteroylglutamic acid of approximately 300 to 1, prevented the "anti-folic" inhibition of the chick oviduct response to estrogenic stimulation. In frogs, however, a folic acid to "4-amino" PGA ratio of 100 to 1 did not prevent the "4-amino" PGA inhibition of the oviduct response to estrogens.[16] It appears clear, therefore, that folic acid may prevent the toxic effects of the "4-amino" folic acids but the protective ratio necessary will undoubtedly differ quantitatively for the specific "anti-folic" compound and the biological system used.

The apparently specific action of 2,6-diaminopurine on blood formation merits further study. It is of interest that Bendich and Brown[17] have shown that 2,6-diaminopurine may serve as a precursor of nucleic acid guanine, an observation which cannot, as yet, be related to the mechanism of blood formation.

Summary. The "4-amino" pteroylglutamic acids profoundly inhibit the growth of the chick embryo, with the production of developmental abnormalities. These compounds are active in the range of 0.003 to 0.005 mg/egg, and their actions are not prevented by large doses of folic acid. The "4-amino" folic acids with aspartic acid, threonine and alanine substituted for the glutamic acid are considerably less toxic but produce similar toxicological effects. Other compounds allied to folic acid are relatively non-toxic and do not possess the growth-inhibitory activity of the "4-amino" compounds.

2,6-diaminopurine, at LD_{50} doses, does not seem to be an active growth inhibitor, but it produces a pale embryo, presumably deficient in hemoglobin.

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[16] Goldsmith, E. D., Schreiber, S. S., and Nigrelli, R. F., PROC. SOC. EXP. BIOL. AND MED., 1948, **69**, 299.

[17] Bendich, A., and Brown, G. B., *J. Biol. Chem.*, 1948, **176**, 1471.

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17221. Distribution of C^{14}, Administered as $CaC^{14}O_3$, in Rat Liver Glycogen and Blood Glucose and Lactate.*

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In prior studies[1-5] it has been shown that the distribution of isotopic carbon in rat liver glycogen following the feeding of a labeled compound may serve as an indicator of intermediary pathways. Under the proper experimental conditions, the distribution of isotope in blood glucose and lactate might reasonably be expected to mirror that in the liver glycogen. Should this expectation be realized, it would be possible to obtain information from the degradation of the carbohydrates from the blood of larger animals and man comparable

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[1] Wood, H. G., Lifson, N., and Lorber, V., *J. Biol. Chem.*, 1945, **159**, 475.

[2] Lifson, N., Lorber, V., Sakami, W., and Wood, H. G., *J. Biol. Chem.*, 1948, **176**, 1263.

[3] Lorber, V., Lifson, N., Wood, H. G., and Sakami, W., *Am. J. Physiol.*, 1948, **155**, 452.

[4] Shreeve, W. W., Feil, G. H., Lorber, V., and Wood, H. G., *J. Biol. Chem.*, 1949, **177**, 679.

[5] Lorber, V., Cook, M., and Bordeaux, J., *Fed. Proc.*, 1949, **8**, 99.

TABLE I.
Distribution of C'^4 in the Degradation Fractions from Rat Liver Glycogen, and Blood Glucose and Lactate.

Exp. No.	Animals wt in g	Total activity administered as CaC'^4O_3 in cts/min.	Activity of 7th day sample of respiratory CO_2 in cts/min/mg C	Activity of the various fractions from blood glucose + lactate, and liver glycogen, in cts/min./mg C				
				Blood glucose + lactate carbon atoms		Liver glycogen carbon atoms		
				3 and 4	1,2,5 and 6	3 and 4	2 and 5	1 and 6
1	328	5.6×10^6	—	24	2	8	0	
2	303	5.1×10^6	100	31	—	5	0	
3	293	2.6×10^7	380	35	0	44	0	1
4	329	2.3×10^7	640	88	0	65	0	3

in significance to that derived from the study of rat liver glycogen. The present paper reports some experiments designed to test this point in the rat.

Methods. Pellets of CaC'^4O_3[6] were implanted intraperitoneally in 4 unfasted white rats. After seven days on stock diet the animals were anaesthetized with intraperitoneal nembutal, and as much blood as possible withdrawn by syringe from the abdominal aorta. Bleeding was facilitated by administering warm Ringer's solution into the inferior vena cava by syringe. The diluted blood, rendered non-clotting by heparin, was placed in a stoppered flask, and held in a water bath at 38°C for 5 hours, with moderate agitation. At the end of this time, practically all the blood glucose was converted to lactate by the glycolytic enzymes of the blood. The blood was deproteinated with metaphosphoric acid, the lactate removed by continuous ether extraction, and degraded as previously described.[1] The acetaldehyde arising from the $KMnO_4$ oxidation of the lactate, representing carbons 1,2,5 and 6 of the glucose, was oxidized without further degradation. The liver glycogen was isolated and degraded by the "bacterial" degradation method described previously.[1] In experiments 1 and 2 the acetaldehyde arising from $KMnO_4$ oxidation of the lactate from the glycogen was precipitated and counted as the 2-4 dinitrophenylhydrazone. In experiments 3 and 4 the aldehyde was further degraded by the iodoform reaction. Daily collections of respiratory CO_2 were made in carbonate free NaOH.

All samples, with the 2 exceptions noted above, were converted to $BaCO_3$ and the activity estimated using an end-window G-M tube. The over-all counting error by the method in use at the time these experiments were carried out was ± 4-5%.

Results and Discussion. The experimental data is summarized in Table I. The results confirm prior findings,[1,4] that isotopic carbon administered as CO_2 appears predominantly in carbons 3 and 4 of the glucose units of liver glycogen. The distribution of isotope in the degradation fractions from blood is qualitatively similar to that found in the glycogen.

The present data must be regarded with certain reservations. The material from blood includes the blood glucose, lactate, and perhaps other related intermediates in trace amounts. The activity, as determined, might, therefore have been contributed mainly by trace substances of very high activity. In view of the fairly low levels of activity noted in the respiratory CO_2, this does not seem likely. The quantitative differences between the fractions from blood and liver glycogen cannot be evaluated with certainty at present.

From the practical point of view, since the method, as applied, yielded degradation fractions from blood that mirrored those from liver glycogen, it should prove a useful tool. Additional experiments are in progress using labeled compounds known to give an isotope distribution pattern in liver glycogen different from that found in CO_2 fixation.

Summary. Experiments are reported in which it was found that CaC'^4O_3 implanted intraperitoneally in rats produced a qualitatively similar isotope distribution pattern in the liver glycogen, and blood glucose and lactate. This result suggests that in isotope feeding experiments, analysis of the blood glucose and lactate may yield information comparable to that obtained by the degradation of liver glycogen. Additional experiments to put this question to a more critical test are in progress.

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[6] Armstrong, W. D., Schubert, J., and Lindenbaum, A., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 233.

It is a pleasure to thank Miss Margaret Cook for valuable technical assistance.

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17222. Activation of Antifungal Extracts of Actinomycetes by Ultrafiltration Through Gradocol Membranes.

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In the course of the purification of extracts from broth cultures of two soil actinomycetes showing antifungal activity, it was observed that solutions which were passed through gradocol membranes often possessed greater activity than the original solutions. Striking results were obtained repeatedly and under varying conditions. Since there may be a wide application in studies of antibiotics, it seems well to report the original observations. The extent and nature of the phenomenon are under investigation.

Materials and methods. Microorganisms. Two distinct actinomycetes, Nos. 47205[1] and 48240, isolated from soil and found to be antagonistic to fungi pathogenic for man, were grown in liquid media dispensed in 250-ml amounts in 1000-ml Erlenmeyer flasks. Strain No. 47205 was grown in glycerol-yeast extract broth containing 0.15% agar for from 9 to 12 days at approximately 28°C, and No. 48240 in glucose-tryptone broth for from 12 to 17 days at the same temperature. The composition of the media varied from the original formulas[1] only in that distilled water replaced the tap water. Four pathogenic fungi, *Candida albicans* No. 4567, *Cryptococcus neoformans* No. 45215, *Trichophyton mentagrophytes* (*gypseum*) No. 45141, and *Trichophyton rubrum (purpureum)* No. 4507 were employed as test microorganisms.

Antifungal tests. The extracts were assayed by the agar-diffusion or cup test and by agar-streak or agar-dilution tests.[2] Glucose-tryptone agar[1] was used throughout. The time of incubation varied according to the rate of growth of the particular test microorganism and the type of test. Thus in the diffusion tests in which *C. albicans* was employed, incubation for 8 to 10 hours was adequate, while with *C. neoformans*, 22 to 24 hours were necessary. The agar-streak tests with all 4 test microorganisms were incubated 72 hours. All tests were incubated at approximately 28°C.

Ultrafiltration. Gradocol membranes were prepared from nitrocellulose in the form of Parlodion according to the method described by Bauer and Hughes.[3] The average pore diameter of the several membranes varied from 52 to 64 mμ.* The solutions were passed through the membranes under positive pressure. After filtration, the membranes were washed by passing through some of the pure solvent. They were then removed from the filter and extracted with a small volume of 95% ethyl alcohol. Whenever the pH of a solution was determined before ultrafiltration, it was in the acid range.

Experimental. Different types of solutions showing antifungal activity were subjected to

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[1] Schatz, A., and Hazen, E. L., *Mycologia*, 1948, **50**, 461.

[2] Reilly, H. C., Schatz, A., and Waksman, S. A., *J. Bact.*, 1945, **49**, 585.

[3] Bauer, J. H., and Hughes, T. P., *J. Gen. Physiol*,. 1934, **18**, 143.

* In one experiment with a coarse membrane, 378 mμ, ultrafiltration did not result in activation of the solution.

TABLE I.
Activation of Antifungal Activity of Butyl Alcohol Extractives of Actinomycete Culture Filtrate by Ultrafiltration.

Actinomycete No. 48240 Test material	Vol., ml	Dilution titer* *C. albicans*	 *C. neoformans*
Original aqueous solution of butyl alcohol extractives†	10	10	640
Ultrafiltrate	Approx. 7	160	>320
Aqueous washings	10	80	320
” ext. of membrane	5	20	80
Alcohol ” ” ”	10	80	160
Original alcohol solution of butyl alcohol extractives†	4.5	40	640
Ultrafiltrate	Approx. 3.5	640	640
Alcohol washings	5.5	160	160
” extract of membrane	4.5	160	80

* Highest 2-fold serial dilution to give a zone of inhibition of at least 10 mm in diffusion or cup test. Figures represent reciprocals of the dilutions.

† Aliquot of butyl alcohol extract of 1/5/49.

TABLE II.
Activation of Antifungal Activity of Ethyl Acetate Extractives of Actinomycete Whole Culture by Gradocol Membranes.

Actinomycete No. 48240 Test material	Vol., ml	Dilution titer* *C. albicans*	 *C. neoformans*
Original alcohol solution	12	<5	1,280
Ultrafiltrate	12	160	20,480
Alcohol washings	12	20	1,280
” extract of membrane	6	160	320
Original sol. shaken with membrane scraps	25	10	10,240
Alcohol extr. of membrane scraps	25	<5	1,280

* Highest 2-fold serial dilution to give a zone of inhibition of at least 10 mm in diffusion or cup test. Figures represent reciprocals of the dilutions.

ultrafiltration. For the initial concentration of active material, whole culture, culture filtrate, or pellicle was extracted with organic solvents; or culture filtrate was treated with charcoal and the charcoal eluted with organic solvents. In each case the organic solvents were removed by distillation *in vacuo* and the residue was dissolved in either water or 95% ethyl alcohol for ultrafiltration.

The effect of ultrafiltration on both a water solution and an ethyl alcohol solution of the butyl alcohol extractives from a culture filtrate of No. 48240 is shown in Table I. Not only was the activity of the ultrafiltrate much greater than that of the solution placed on the filter, but the alcohol extract of the membrane showed marked activity, especially against *C. albicans.*

In another experiment an alcohol solution of the ethyl acetate extractives of whole culture was divided into two portions: one was passed through a gradocol membrane and the other shaken with small scraps of membrane. The results are presented in Table II. Both solutions were activated; but the activity in the one filtered through the membrane was greater, particularly against *C. albicans.* In the original solution no activity against *C. albicans* was demonstrated in a 1:5 dilution; after ultrafiltration the solution inhibited growth in a dilution of 1:160. When filtrates of the broth cultures of the actinomycetes were treated with charcoal (Darco G 60) to remove the active agent and the charcoal was eluted with water or organic solvents, no activity against *C. albicans* could be demonstrated by diffusion tests. Ultrafiltration, however, resulted in activation of these eluates against *C. albicans* to a dilution of 1:640. The protocol of such an experiment is given in Table III.

Enhancement of activity was not peculiar

TABLE III.
Activation of Antifungal Activity of Charcoal Eluates of Actinomycete Culture Filtrate by Ultrafiltration.

Actinomycete No. 48240 Test material	Vol., ml	Dilution titer* C. albicans	 C. neoformans
Original alcohol solution of charcoal eluates	10	0	80
Ultrafiltrate	9	640	640
Alcohol washings	10	40	40
" extr. of membrane	5	320	320

* Highest 2-fold serial dilution to give a zone of inhibition of at least 10 mm in diffusion or cup test. Figures represent reciprocals of the dilutions.

TABLE IV.
Activation of Antifungal Activity of Extractives from Actinomycete Culture Pellicle by Ultrafiltration.

Actinomycete No. 47205 Test material	Vol., ml	Dilution titer* C. albicans	 C. neoformans
Original alcohol solution	10	<5	>320
Ultrafiltrate	Approx. 7.5	320	640
Alcohol washings	10	40	160
" extr. of membrane	5	160	160

* Highest 2-fold serial dilution to give a zone of inhibition of at least 10 mm in diffusion or cup test. Figures represent reciprocals of the dilutions.

to a single actinomycete nor to one culture medium. Similar results obtained with No. 47205 are shown in Table IV. In this case extractives obtained from the pellicle with ethyl acetate were chromatographed on alumina and an alcoholic eluate subjected to ultrafiltration. The original solution was without activity against *C. albicans* but the ultrafiltrate inhibited growth in a dilution of 1:320. By the agar-streak test enhancement of antifungal activity of the ethyl acetate extractives of No. 48240 culture filtrate was also demonstrated against *C. albicans, C. neoformans, T. gypseum*, and *T. purpureum*. (Table V).

Water or alcohol ultrafiltrates of gradocol membranes are inactive indicating that the phenomenon is not due to a principle in the filter itself.

Discussion. It is not uncommon for the activity of antibiotic substances to be reduced or lost unaccountably during the course of purification. A few such observations that seem to be more or less related to our own are noted briefly. Woodruff and Foster[4] traced the loss of antibacterial activity in bacillin to the presence of an inhibitor of peptide nature that occurs naturally in many complex organic substances. Cavallito and others[5] found that sulfhydryl compounds had an inactivating effect upon some antibiotics. In an examination of the separation of penicillins by the method of counter-current distribution, Craig and his colleagues[6] observed that the use of ethyl acetate led to a certain degree of inactivation. They suggested that this might be due to a particular lot of ethyl acetate. Another observation that may be related was made by Junowicz-Kocholaty and others:[7] namely, that in the purification of sulfactin, activity apparently disappeared in butyl alcohol extracts, but reappeared subsequently in chloroform extracts. On the other

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[4] Woodruff, H. B., and Foster, J. W., *J. Bact.*, 1946, **51**, 371.

[5] Cavallito, C. J., Bailey, J. H., Haskell, T. H., McCormick, J. R., and Warner, W. F., *J. Bact.*, 1945, **50**, 61.

[6] Craig, L. C., Hogeboom, G. H., Carpenter, F. H., and duVigneaud, V., *J. Biol. Chem.*, 1947, **168**, 665.

[7] Junowicz-Kocholaty, R., Kocholaty, W., and Kelner, A., *J. Biol. Chem.*, 1947, **168**, 765.

TABLE V.
Activation of Antifungal Activity by Ultrafiltration Demonstrated by Agar-streak (Agar-dilution) Tests.

	Growth of test fungi							
	C. albicans		*C. neoformans*		*T. gypseum*		*T. purpureum*	
Dilution of material	Original*	Ultra-filtered	Original*	Ultra-filtered	Original*	Ultra-filtered	Original*	Ultra-filtered
1:40	4+		—		—		—	
1:80	4+	—	—	—	±	—	±	—
1:160	4+	—	—	—	2+	—	2+	—
1:320	4+	—	—	—	3+	—	3+	—
1:640	4+	—	—	—	3+	—	3+	—
1:1,280	4+	2+	±	—	3+	—	3+	±
1:2,560		3+		2+		—		±
Controls	4+		4+		4+		4+	

* The ethyl alcohol solution of ethyl acetate extractives from No. 48240 culture filtrate.
— = No visible growth.
± = Least detectable growth.
+ = Scanty, but definite growth.
2+ = Moderate growth.
3+ = Good growth.
4+ = Maximum growth.

hand, Melin and Wikén[8] reported that aqueous extracts of pure forest litter that were somewhat active against staphylococci became more active when autoclaved or passed through a Seitz filter pad.

Whatever the explanation of the present results, it is evident that the ultrafiltration technic may be applied profitably in the study of antibiotics.

Summary. The antifungal activity of some partially purified extracts of two actinomycetes was sharply increased by filtration through gradocol membranes.

We wish to thank Mr. James Quigley for carrying out all the procedures in ultrafiltration.

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[8] Melin, E., and Wikén, T., *Nature*, 1946, **158**, 200.

17223. Relation of a Specific Strain of Salmonella to Ulcerative Cecitis of Rats.

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Although *Salmonella enteritidis* is found very constantly in the lesions of ulcerative cecitis of rats[1] some doubt remains as to whether this organism is the sole cause of the disease. The possibility that some other agent, perhaps a virus, may be concerned is suggested by certain findings; the arguments pro and con can be summarized briefly as follows:

(1) *Arguments in favor of Salmonella being the specific etiological agent in cecitis of rats.* a. *Salmonella enteritidis* has been found by all workers to be invariably associated with the lesions.[2] b. Salmonella may be obtained from the enlarged mesenteric lymph nodes associated with cecitis.[2] c. Cecitis may be prevented by rations containing either sulfaguanidine or sulfasuxidine.[3] d. The le-

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[1] Buchbinder, L., Hall, L., Wilens, S. L., and Slanetz, C. A., *Am. J. Hyg.*, 1935, **22**, 119.

[2] Bloomfield, A. L., and Lew, W., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 179.

sions can be cured by ingestion of streptomycin which inhibits (*in vitro*) in high dilution the strains of salmonella recovered from the cecum.[4] e. Attempts to isolate a virus have so far been fruitless.[5]

(2) *Arguments which suggest that some other agent may play a part.* a. The anatomy of the lesions—early involvement of lymph nodes and later localized granulomatous lesions are not known to be caused by members of the salmonella group. b. The large cysts containing clear fluid so often found as part of the cecitis picture do not yield salmonella or other organisms by ordinary culture technic.[2] c. Salmonella isolated from the lesions and fed in large dosage to healthy rats produce a violent diffuse enteritis, rather than the usual picture of cecitis.[2] d. Some of the animals which recovered from such an acute enteritis were later found to have typical lesions of chronic cecitis but with no greater frequency than uninoculated controls.[2] e. Cecitis in the offspring can be prevented by treatment of the mothers with sulfonamides during pregnancy and lactation. It is not clear why such a procedure should protect against salmonella infection.[6] f. Rats fed a diet deficient in Vitamin B develop cecitis less often than controls given a normal diet.[7] This result suggests the possibility of a virus infection and is just the opposite of what one would expect with salmonella.

Experiments. The following studies were therefore made in the attempt to define more precisely the role of salmonella in chronic ulcerative cecitis of rats. The first group of experiments was designed to determine relation of the development of lesions to the presence of salmonella and to the titer of specific antibodies.

Seven groups of 6 young rats were used. Each group was housed in a separate cage and was fed the stock diet of the laboratory as desired. At the age of 2 months and thereafter at intervals of 2 weeks one group was sacrified. Under ether anaesthesia and with aseptic technic the abdomen was opened and the cecal region was carefully examined for suspicious lesions. The cecum was then excised, opened, and searched for ulceration. Material for culture was taken from the cecum, the swab being thoroughly rubbed over the mucosa. Except in the case of the first 2 groups the animals were then exsanguinated by cutting the abdominal vessels from which blood was collected for serological tests.

Stool cultures were made on MacConkey (Difco) and salmonella-shigella (Difco) agar plates, from which colonies were sub-cultured in Russell double sugar tubes and in liquid carbohydrate media. Various strains were finally tested with salmonella diagnostic sera (Lederle). Sera to be tested for agglutinins were stored in the refrigerator. Serial dilutions were set up against a live suspension of 24 hour growth of the salmonella isolated from cecal lesions. The tubes were held for 2 hours in the water bath at 37°C placed over-night in the refrigerator and then read at room temperature.

Results. The results are summarized in Table I. Aside from the normal bowel flora the only organisms isolated were members of the salmonella group. These were obtained from 9 animals among a total of forty-two. In each case the characteristics were identical and conformed to *Salmonella enteritidis*, Group D of the Kauffmann-White Schema. Furthermore 6 strains which were identified in the salmonella typing laboratory of the State Department of Public Health all had the same antigenic formula: – (I), IX, XII . . . : g . . . ; —

A study of Table I shows several points of interest. There was no visible cecitis in the 2 to 3 months old groups. From 3½ months on, with the exception of the 4 months group, cecitis was evident in approximately one-half the animals. Organisms of the salmonella group were recovered in only one instance in which gross lesions were not evident, (Rat I of Group IV), and then only

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[3] Bloomfield, A. L., and Lew, W., *ibid.*, 1941, **48**, 363; 1942, **51**, 28.

[4] Bloomfield, A. L., and Lew, W., *ibid.*, 1948, **69**, 11.

[5] Schultz, E. W., personal communication.

[6] Bloomfield, A. L., and Lew, W., *Am. J. Med. Sci.*, 1943, **205**, 383.

[7] Bloomfield, A. L., and Lew, W., *J. Nutrition*, 1943, **25**, 427.

TABLE I.
Relation of Lesions of Cecitis to Presence of Salmonella, and Titer of Agglutinins in Rats of Various Ages.

Rat. No.	Lesions	Culture Salmonella	Agg. titer
	Group I—2 mo.		
6 rats	None	All negative	Not done
	Group II—2½ mo.		
6 rats	None	All negative	Not done
	Group III—3 mo.		
6 rats	None	All negative	4, 0, 0, 0, 4, 8
	Group IV—3½ mo.		
Rat No. 1	No cecitis	10	32
2	Walls of cecum seem a little thick, no ulcer	50	8
3	No ulcer seen but wall a little thick and there is a mesenteric cyst 0.5 cm	10	32
4	No cecitis	0	0
5	Frank cecitis with ulcer 1 cm in diam.	Innumerable	32
6	No cecitis	0	0
	Group V—4 mo.		
6 rats	None	All negative	8, 4, 4, 4, 4, 0
	Group VI—4½ mo.		
Rat No. 1	No cecitis	Negative	8
2	” ”	”	8
3	Marked thickening of cecum with ulcer 0.5 cm, large cysts and adhesions	Many	16
4	No cecitis	Negative	32
5	” ”	”	64
6	Typical ulcer 0.5 cm	Many	8
	Group VII—5 mo.		
Rat No. 1	No ulcer, thickening of cecal wall and cyst 0.5 cm	A few	32
2	Extreme lesions, huge ulcer	Innumerable	32
3	No cecitis seen	Negative	8
4	” ” ”	”	16
5	Marked lesion, ulcer 1 cm	Innumerable	16
6	No cecitis seen	Negative	0

a few colonies were obtained. The number of salmonella usually paralleled the extent of the lesions; when the lesion was slight a few up to 50 organisms per plate were obtained, when the lesion was advanced there were always "many" to "innumerable" colonies. The point of greatest importance however, is that as rats grow and develop cecitis salmonella are already present in the earliest lesions; they do not appear only after cecitis is advanced.

The agglutinins are of special interest. Among the 8 animals with visible cecitis 2 had a titer of 8, two had a titer of 16, and 4 had a titer of 32. Among 21 animals without visible lesions and with negative cultures for salmonella, however, 14 showed titers varying from 4 to 64. Furthermore, one-half the animals in the 3 month old group showed agglutinins at a time when no cecitis was yet to be seen and no cultures were positive.

It is clear then that there is an early and frequent, although slight, immunological reaction to salmonella enteritidis in these rats at a time before lesions of cecitis are visible, and before it is possible to isolate salmonella by our technic. The question was pursued further by examining the sera from even younger

TABLE II.
Agglutinin Titer Against *Salmonella enteritidis* of Young Healthy Rats.

Age 2 months		Age 1 month		Age 12 days	
Rat No.	Titer	Rat No.	Titer	Rat No.	Titer
2A-F	8, 4, 16, 4, 8, 16	1A-F	8, 8, 32, 8, 8, 4	1-6	0, 4, 0, 4, 8, 8
				Mother	8

rats. All the animals were healthy and none showed cecitis. Six were 2 months old, 6 were one month old, and 6 were 12 days old. The eyes were not yet open in the last group and they had not yet been weaned. Serum from the mother of this last group was also tested. The results are shown in Table II. It is seen that even the younger rats may already show specific agglutinins against salmonella at a time long before cecitis can be demonstrated or salmonella isolated. The presence of such agglutinins are therefore of little diagnostic significance and it will be of interest to look for similar antibodies in a rat colony entirely free from cecitis.

Further evidence for the causal role of salmonella was then sought in the following experiments. Sixty rats about 2 months old were divided into 3 groups of 10 test animals and three groups of litter-mate controls. On 5 consecutive days one solid loop of growth from a 24 hour agar slant of salmonella enteritidis isolated from rats with cecitis was shaken up in the water bottles of the test animals. Culture of the water thereupon showed heavy growth. None of the animals however, developed any clinical indisposition with these doses and they all gained weight normally. After one, 2, and 3 months when the rats were 3, 4, and 5 months old one set of test animals with its controls were sacrificed under ether anaesthesia and the degree of cecitis, if any, was noted. The results are shown in Table III. It is seen that one month after ingestion of salmonella there was very little cecitis in either test animals or controls. After 2 months definite cecitis was present in some animals, a little more in the test group than in the controls; but after 3 months cecitis was rampant in the culture-fed animals with not a single definite lesion in the controls.

Discussion. The above experiments were designed to define more exactly than has heretofore been done the significance of *Salmonella enteritidis* in cecitis of rats. The invariable relationship of salmonella to the lesions was confirmed; no culture from a visible lesion, even very early, failed to yield the specific organism. On the other hand salmonella were never recovered from the cecum unless a lesion was present except in one instance (animal No. 1 of the 3½ months group) and then only a few colonies were isolated. Specific agglutinins were uniformly present in the sera from animals with cecitis, but since such agglutinins were frequently present without either cecitis or a positive culture for salmonella, the significance of these antibodies is uncertain. On the whole higher titers were obtained from animals with frank lesions than from healthy rats.

These observations demonstrate conclusively that no cecitis occurs in this colony without the presence of the specific salmonella. However, this finding does not entirely exclude the association of some other unidentified agent in producing the infection. The inoculation experiments are of interest in this connection. Rats fed salmonella did in the end show much more cecitis than untreated controls but the lesions developed only after what appeared to be a long latent period of from one to 3 months. Where are the specific bacteria during this long interval? There is some suggestion that the earliest lesion of the cecitis syndrome is an adenitis of the regional nodes, (Table III) and that the actual cecal lesion only develops after a considerable interval. If this is true the long latent period may be explained, but such a course of events is unknown with salmonella infections. We plan to explore this possibility in further studies. Meanwhile it seems clearly established that ingestion of the specific salmonella promoted the development of cecitis which in turn never occurred in the absence of the

TABLE III.
Incidence of Cecitis in Rats Fed Salmonella Enteritidis, and in Untreated Controls.

Degree of cecitis in rats aged 3 mo.		Degree of cecitis in rats aged 4 mo.		Degree of cecitis in rats aged 5 mo.	
Test animals	Controls	Test animals	Controls	Test animals	Controls
0	0	0	0	0	0
0	0	0	0	0	0
0	0	0	0	0	0
0	0	0	0	?	0
?	0	0	0	++	0
?	0	?	0	+++	0
?	0	?	0	+++	0
?	0	++	0	+++	0
+	0	++	?	++++	0
+	?	++	++++	++++	?

0 = No ulcer, no glands, no cysts.
? = No ulcer, slight or doubtful enlargement of glands, no cysts.
+ = Ulcer to 0.5 cm, enlarged glands and/or cysts.
++ = Ulcer 0.5 to 1.0 cm, enlarged glands and/or cysts.
+++ = Ulcer 1.0 to 2.0 cm, enlarged glands, cysts, adhesions, and matting.
++++ = Huge ulcers, enlarged glands, cysts, adhesions, and matting.

organisms. Of special interest is the fact that in spite of the extensive infestation of the colony healthy carriers are of greatest rarity.

Summary. 1. A specific strain of salmonella has been invariably obtained on culture from the ulcers of rat cecitis. 2. Salmonella were not obtained from the cecum in the absence of lesions with one exception. 3. Agglutinins for the specific strain of salmonella are found in most of the rats in this colony although the titer is on the average higher in animals with visible cecitis. 4. Feeding the specific salmonella in appropriate doses was followed after a long latent period by development of cecitis in many of the test animals. 5. The association of some other synergistic agent is not fully excluded but seems at present unlikely.

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17224. Aminopterin and Response of Frog Oviducts to Estradiol. II. Histological Studies and Mitotic Counts.[*]

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In a previous paper,[1] the gross changes in the immature female frog oviduct following parenteral administration of estradiol benzoate[†] alone and in conjunction with folic acid[‡] and with a folic acid antagonist, aminopterin,[‡] were described. Aminopterin (4-amino pteroylglutamic acid) inhibited the growth and differentiation response of oviducts of newly metamorphosed female frogs, *Rana clamitans,* to estradiol. The aminopter-

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[*] This investigation was supported by a research grant from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service.

[1] Goldsmith, E. D., Schreiber, S. S., and Nigrelli, R. F., PROC. SOC. EXP. BIOL. AND MED., 1948, **69**, 299.

[†] The estradiol benzoate was generously supplied by Dr. F. F. Yonkman and Dr. F. L. Mohr of Ciba Pharmaceutical Products.

[‡] The 4-amino pteroylglutamic acid was generously supplied by the late Dr. Y. SubbaRow, and the folic acid by Dr. T. H. Jukes and the late Dr. A. L. Franklin of Lederle Laboratories.

in effect could not be overcome by folic acid in concentrations as high as 100 parts of folic acid to 1 part of aminopterin. In the light of more recent work, it is probable that pre- and concurrent treatment with higher dosages of folic acid would have reversed the antagonist action.

Animals were fixed in Bouin's solution and serial sections of oviducts extending along the craniad half or two-thirds of the kidney were cut at 7 μ and were stained with hematoxylin and eosin, iron hematoxylin, or with Masson's stain. Mitotic counts were made of the oviduct epithelium; sections from each slide were examined so that comparable areas in all the animals were studied in order to obtain estimates of mitotic activity throughout the length of the oviduct.

Results. In the untreated controls, the small and uncoiled oviducts consisted of a single layer of cuboidal epithelium with regular oval or round nuclei which encircled a small lumen. Mitotic counts of the oviduct epithelium revealed less than one mitotic figure per thousand cells. After the administration of estradiol for 2 to 3 weeks (0.1 mg per week), there was marked increase in the diameter, length and coiling of the oviduct. The bulk of the oviducts consisted of glands which radiated from the oviduct lumina to the periphery. The gland cells contained basal nuclei and at times granular cytoplasm. The lumina were lined with well differentiated ciliated columnar epithelium with regular oval, vesicular nuclei. Mitotic figures were rare, and in over 12,000 cells the incidence of mitotic figures was less than one per 1500 cells. Addition of folic acid to estradiol accentuated the growth and differentiation response of the oviducts, but the mitotic counts were not significantly different from those animals receiving only estradiol.

Either pretreatment with aminopterin or simultaneous treatment of the frogs with aminopterin and estradiol appeared to inhibit the estrogen response. The oviducts remained uncoiled, increased in diameter only slightly and showed areas of cystic dilatation. There was no evidence of gland formation, and a single layer of low columnar epithelium lined the lumina which were only slightly larger than those in untreated controls. In the areas of cystic dilatation, the epithelium around the lumina was flat and almost squamous in appearance. There was a significant increase in mitotic figures in these oviducts, with 12 to 36 figures per thousand cells. Although metaphases predominated, there were considerable numbers of the other mitotic phases.

Except for some slightly increased infolding of the oviduct epithelium into the lumina, supplementation of the aminopterin and estradiol with folic acid did not significantly change the histological picture characteristic of those receiving only the antagonist and estradiol. Mitotic counts in all but one animal revealed 17 to 46 mitotic figures per thousand cells. In 2 animals, one receiving aminopterin and estradiol, and the second receiving folic acid as well, where there was no significant increase in mitoses over the controls, the nuclei of the oviduct epithelium were markedly abnormal, with great variations in size and shape. The rest of the histological picture was no different from other animals treated similarly.

Discussion. The results indicate that although the growth and differentiation of the oviducts were inhibited by aminopterin the number of mitotic figures was significantly increased. It must be emphasized that the administration of estradiol alone is followed by an increase in the number of cells and mitoses. However, at the time of sacrifice, 2 to 3 weeks after initiation of treatment, the most rapid growth had passed its peak. At the end of this period, the oviducts under the influence of the antagonist and the estrogen had grown but slightly, but a large number of mitotic figures were present.

This apparently paradoxical situation of a picture of numerous mitotic figures and very little growth may be resolved by the following considerations.

From the observations of Brachet[2] and Painter,[3] Koller[4] has adduced that the amount of available nucleic acid is one of the principal factors which determine the rate of cell division. It was suggested[5] that folic acid may function as a coenzyme in a system responsible for the synthesis of thymine or a thymine-like compound which is then utilized in the synthesis of nucleic acid. Further evidence of this relationship is to be seen in *Lactobacillus casei* which when grown in a folic acid deficient medium showed a marked decrease in desoxyribonucleic acid,[6] a universal constituent of cell nuclei.[7]

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[2] Brachet, J., *Arch. Biol.*, Paris, 1940, **51**, 151.

[3] Painter, T. S., *Proc. Nat. Acad. Sci.*, 1940, **26**, 95.

[4] Koller, P. C., *Nucleic Acid*, Cambridge Univ. Press, 1947, p. 280.

It is tempting to speculate that in the presence of aminopterin, a folic acid antagonist, the folic acid utilization in the organism may be interfered with, resulting in a reduction in nucleic acid synthesis and a marked retardation of the rate of cell division. These facts might serve to explain the growth inhibition obtained with the folic acid antagonists in certain of the leukemias[8,9] and in the transplantable mouse Sarcoma 180.[10]

Summary. Macroscopically and microscopically, oviducts of frogs receiving aminopterin (4-amino pteroylglutamic acid) failed to respond to the growth stimulating action of estradiol. However, a significantly greater number of mitotic figures were visible in these oviducts. Evidence is presented to suggest that the folic acid antagonists may exert their growth inhibitory action by interfering with folic acid utilization in the organism, with a consequent reduction in nucleic acid synthesis and a retardation of the rate of cell division.

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[5] Stokes, J. L., *J. Bact.*, 1944, **48**, 201.

[6] Prusoff, W. H., Teply, L. J., and King, C. G., *J. Biol. Chem.*, 1948, **176**, 1309.

[7] Stedman, E., and Stedman, E., *Nucleic Acid*, Cambridge Univ. Press, 1947, p. 232.

[8] Farber, S., Diamond, L. K., Mercer, R. D., Sylvester, R. F. Jr., and Wolff, J. A., *New England J. Med.*, 1948, **238**, 787.

[9] Burchenal, J. H., Burchenal, J. R., Kushida, M. N., Johnston, S. F., and Williams, B. S., *Cancer*, 1949, **2**, 113.

[10] Moore, H. E., Stock, C. C., Sugiura, K., and Rhoads, C. P., PROC. SOC. EXP. BIOL. AND MED., 1949, **70**, 396.

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17225. Increased Metabolic Rate in Rats after X-Irradiation.

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Some metabolic reactions may be increased after exposure to total-body irradiation. Indirect evidence for increased metabolism in dogs 1-3 weeks after irradiation was presented by Prosser *et al.*:[1] (1) Weight loss was greater after irradiation than in non-irradiated dogs in which food intake was limited to amounts less than consumed by the irradiated animals. (2) Nitrogen excretion was maintained by irradiated dogs despite a decrease in food intake, and in the acute terminal period the nitrogen balance was negative. (3) The metabolic balance minus the water balance or metabolic index is given by [(food + water + weight loss) − (urine + feces)] − [(food water + water drunk) − (urine + feces water)]. This metabolic index is a measure of CO_2 exhaled plus water of metabolism and water of tissue breakdown. This quantity increased significantly in dogs during the first 2 weeks after irradiation and increased considerably during the acute terminal period.

As a check on the hypothesis that catabolism is increased after irradiation, basal metabolism has been measured in rats after total body exposure to x-rays.

Materials and methods. Forty-four albino rats, 24 males and 20 females, ranging in initial weight from 160 to 310 g, (average 206 g) were used. There was no apparent dif-

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* Present address: Physiology Department, University of Wisconsin, Madison, Wis.

[1] Prosser, C. L., with contributions by Painter, E. E., Lisco, H., Brues, A. M., Jacobson, L. O., and Swift, M. N., *Radiology*, 1947, **49**, 299.

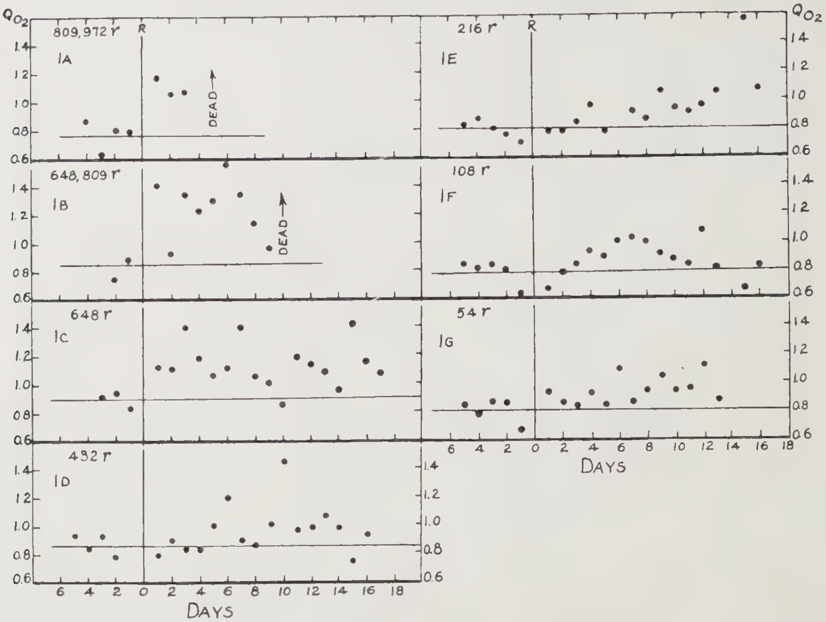

FIG. 1a-g.

Average oxygen consumption in ml O_2/g/hr of groups of rats irradiated at day R. 1a: 6 rats which died 3-5 days after exposure; 1b: 8 rats which died 8-10 days after exposure; 1c: 6 rats which survived potentially lethal doses; 1d: 4 rats exposed to 432 r. 1e: 5 at 210 r. 1f: 5 at 108 r. 1g: 4 at 54 r. Horizontal lines: Control average Q_{O_2} for each group.

ference in reaction as a function of size or sex. The postabsorptive oxygen consumption was measured daily between noon and 4 P.M.; animals were weighed after each metabolism measurement.

The equipment used for measurement of oxygen consumption was a modification of the apparatus described by Schwabe and Griffith.[2] This is a closed gas circuit consisting of a glass animal chamber, a circulating oil-immersed pump, CO_2 and H_2O absorbers, and two flasks arranged as water valves which maintain a constant pressure in the entire system. Oxygen is supplied through the pressure-maintaining flasks from a rubber bag. Volume differences in the first of the pressure flasks are transmitted to a water manometer from which a float writes on a kymograph. Movement of the animal was reflected in a steeper rise of the kymograph record. All measurements of Qo_2 were based on the least slope, (which corresponded to periods when the animal was at rest) and were expressed as ml of O_2 consumed per g body weight per hour. Animals were placed in the chamber for a half hour each day until they became accustomed to the apparatus. Then control measurements were made for several days prior to irradiation.

The rats were irradiated, 2 to 3 at a time, in a cell cut into a block of presswood. The dimensions of the block were 40 x 40 x 20 cm, those of the cell 18 x 18 x 10 cm. One half of the dose was administered from above, one half from below. Factors were: 200 K.V.p., no filter, T. D. of 55½ cm (to center of cell). The dosage distribution was determined by measurement with a Victoreen thimble chamber in a phantom of presswood equal in size to the irradiation block and cell. It was found that the dosage distribution was fairly uniform, the minimal dose (at the corners of the cell) being 83% of the maximal dose (in the center). The average tissue dose rate was 40.5 r per minute.

Experimental results. The animals have been divided into 4 groups, in a way that was

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[2] Schwabe, E. L., and Griffith, F. R., *J. Nutrition*, 1938, **15**, 187.

TABLE I.
Oxygen Consumption in Contol Animals: Analysis of Variance.

	Sum of squared deviations	Degrees of freedom	Variance	Standard deviation
Daily variations	0.39	55	0.007	0.08
Var. between rats	0.35	17	0.021	0.14
Total variation about the general mean = 81	0.74	72	$\frac{\delta 1}{\delta 2}$ 1.75;	$P < 0.0027$

suggested by a survey of the responses obtained. Of 20 rats which received doses of from 648 to 972 r, 6 died 3 to 5 days, 8 died 9-10 days after treatment, and the remaining 6 survived. The clustering of survival times around 2 values suggests 2 groups defined by survival times. That deaths occur at certain times after irradiation has been frequently observed; the clinical syndrome is different according to the time of death. The third group contains the rats which survived a potentially lethal dose. In the fourth group are animals treated with sublethal doses. The group averages of Qo_2 values are presented in Fig. 1.

Controls. Measurements of oxygen consumption before irradiation yielded an average of 0.83 ml/g/hr which is slightly higher than values given by Moses.[3] The variance was analyzed, following R. A. Fisher's procedure,[4] on the material obtained from 17 rats on each of which 4 to 5 control measurements had been made. The data are given in Table I which shows that the oxygen consumption of any single rat varies from day to day with a standard deviation of about 10% of the mean, and that differences between rats are higher than expected on the basis of random sampling. The excess standard deviation is about 15% of the mean.

Death in 3 to 5 days. Six of 12 rats which received 972 or 809 r developed severe diarrhea, lost weight rapidly, and died 3 to 5 days after exposure. Their Qo_2 was increased by about 35% within 24 hours after treatment (earlier measurements were not taken) (Fig. 1a). These animals lost an average of 17% of body weight by the time of death (Fig. 2a).

Death in 9 to 10 days. Six of the 12 rats irradiated with 809 or 972 r, and 2 out of 8 treated with 648 r, died 9 to 10 days after irradiation. No severe diarrhea occurred in this group. Their Qo_2 was elevated at the first measurement after exposure and remained—on an average—58% higher than the preirradiation level until the day before death, when it declined (Fig. 1b). Weight loss was not rapid in this group; it reached 17% at 5-6 days (Fig. 2b). Thereafter, there was little change in weight, some individuals showing a slight gain. Weight loss definitely did not mirror the course of the metabolic rate.

Survival after potentially lethal doses. Six of 8 rats which received 648 r survived beyond the time when acute death occurs. Each of these showed an increase in oxygen consumption. The average increase was 32% during the first 7 days. During the period of 8 to 10 days after the irradiation—at the time when the second group of non-survivors was dying—there was in some individuals a depression of the metabolic rate to only 10% above the preirradiation level. After this brief period, higher Qo_2 values (on the average 25% above normal) were again obtained. At 17 days, oxygen consumption was still elevated (Fig. 1c). The weight loss in this group reached about 7% on the fifth day. The weight remained at this level until about the ninth day, after which it rose toward the preirradiation level (Fig. 2c).

Sublethal doses. Doses of 432, 216, 108, and 54 r were administered to 4, 5, 5, and 4 rats respectively. With all 4 doses, the metabolic rate rose slightly (an average of 8%) for a 16-day period following irradiation. The difference is small but highly significant. It

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[3] Moses, L. E., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 54.

[4] Fisher, R. A., Statistical methods for research workers, 10th ed., 354 pp., N. Y., G. E. Stechert Co., 1946.

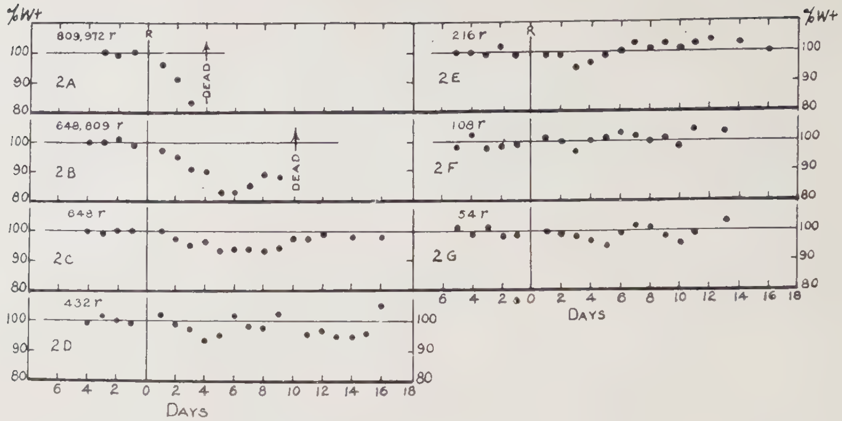

FIG. 2a-g.

Average percentage of body weight before and after irradiation at time R. For each group of rats 100% corresponds to the mean pre-irradiation weight. Doses as indicated.

is based on 74 tests before and 199 tests after irradiation, in a total of 18 rats. The standard deviation of this difference in metabolism between the rats before and after irradiation is about 0.014 or 1.7% of the normal metabolic rate. This is only one-fifth of the difference observed between pre- and postirradiation metabolism. An inspection of the curves (Fig. 1d to 1g) suggests that the rise of the metabolic rate occurs more slowly at these lower doses than in the 3 groups previously described in the lethal range, and that the response does not vary much over the dosage range from 54 to 432 r. Our material is not large enough to warrant any definite assertion beyond the general statement that the metabolic rate does increase after administration of small doses. Weight loss, if it did occur at all, was small and of short duration in the sublethal range (Fig. 2d to 2g).

Tests with nembutal. Eight of the experimental rats were lightly anaesthetized with 0.05 g nembutal per 100 g body weight each day before their metabolic rate was measured The average Qo_2 of the nembutalized animals was 0.79 compared to the control value of 0.82 in 25 unanaesthetized animals tested at about the same time. The response of the metabolic rate to irradiation was about the same in both sets of animals.

Discussion. The preceding data show that total body irradiation produces increased oxygen consumption in rats. This reaction occurs over the whole dosage range tested, from 54 to 972 r. The increase is pronounced after lethal doses—whether or not the animal survives—and slight but still significant after sublethal doses. The rise occurs during the first postirradiation day after lethal doses; it seems to develop more slowly after sublethal doses.

The increased oxygen consumption is certainly not due to excessive feeding; irradiated animals eat less than normal. Neither is it due to increased activity, as shown by frequent observations of activity and by the nembutal tests. After high doses, the metabolic rate remains high during a time when the animals are recovering their normal weight, and after small doses the metabolic rate may go up without associated weight loss. Loss of body weight and increase in metabolic rate depend upon consumption of body substance, but there are probably several factors which influence only metabolic rate or body weight and some of these must be responsible for the observed lack of conformity between the two kinds of measurement.

The increase observed in metabolism of the rat after irradiation need not be interpreted as direct stimulation of oxidative enzyme systems. In fact, all available evidence suggests that the metabolic response may result from indirect action. Measurements in buffered saline of respiration of tissue slices from irradiated animals show either a depression or no change.[5] Similarly respiration is reduced in tissues and isolated enzymes irradiated *in vitro* (Barron *et al.*,[5] Dale[6] and others). Apparently the response of an intact animal where the tissues are bathed by the complex body fluids differs from the response of tissues in simpler media. The body fluids of irradiated animals contain products of tissue breakdown, and there is much evidence of toxemia during the acute radiation reaction. It is not likely that all the toxin is liberated at the time of irradiation. It is more probable that some cell injury remains latent for days and results, over an extensive period, in production of materials which have a specific dynamic action, reflected in elevated metabolism. It is of interest that thyroid metabolism, as indicated by the iodine turnover, is increased 2 to 3 days after irradiation.[7]

Summary. 1. Rats which died 3 to 5 days after total-body irradiation of 809 - 972 r showed an elevation of oxygen consumption of about 35% occurring within 24 hours.

2. Rats which died 9 to 10 days after doses of 648 to 972 r showed an increase in metabolism of about 58% and a decline prior to death.

3. Rats which survived doses of 648 r showed an average increase in oxygen consumption of 32% during the first week, and less pronounced increase extending over a period of at least 16 days after irradiation.

4. At doses from 54 to 432 r there was an increase in metabolism by about 8½%. The maximum oxygen consumption was reached later at sublethal rather than at lethal doses.

5. Increased metabolism did not coincide with weight loss either in time course or in dose dependence.

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[5] Barron, E. S. G., to be published in *Nat. Nucl. En. Ser.*, 1949, IV. 22B.

[6] Dale, W. M., *Brit. J. Rad.*, 1943, **19**, 171.

[7] Evans, T. C., Clarke, G., and Sobel, E., *Anat. Rec.*, 1947, **99**, 21.

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17226. Differential Blood Counts on Rats During Shock Induced by Tourniquets.

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The hematological changes which occur during secondary shock have been reported by Cannon, Frazer, and Hooper,[1] and other workers as reviewed by Moon.[2] Among the changes reported are variations in the red and white cell counts, but the relative values of the different white cells in particular have been neglected. White and Dougherty[3] have shown that in instances of stimulus or stress there is a dissolution of lymphocytes. It is the purpose of this study to show the changes which occur in the percentages of lymphocytes and polymorphonuclear neutrophils during induced experimental shock. Through the course of the experiment, it was found that the changes in the percent of monocytes, basophils, and eosinophils were too small to be significant.

Materials and methods. Albino rats, Sherman strain, including both sexes, and ranging in weight from 98 to 292 g were used. Rubber bands, tightly wrapped, were placed at the highest possible point on both thighs. The rats were kept in a semi-conscious state with intraperitoneal injections of nembutal (Abbott). Blood was obtained by puncturing a tail vein and then subjected to the usual methods for determining gross and differential counts.

Preliminary experiments on 12 rats were

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[1] Cannon, W. B., Frazer, J., and Hooper, A. N., *J.A.M.A.*, 1918, **70**, 526.

[2] Moon, V. H., *Shock*, 1942, Lea and Febiger, Philadelphia, Pa.

[3] White, A., and Dougherty, J. F., *Ann. N. Y. Acad. Sci.*, 1946, **46**, 859.

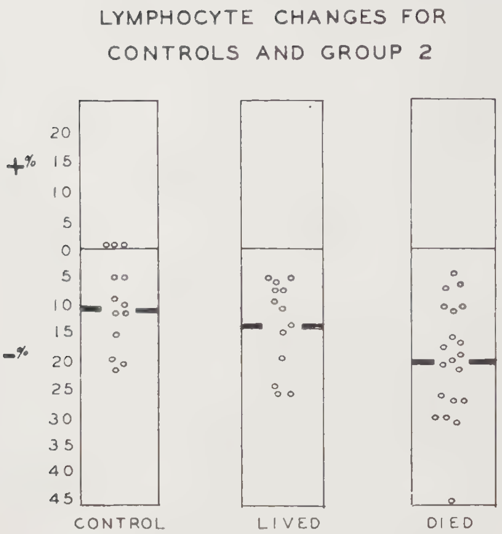

Fig. 1.
Each circle represents the change in lymphocytes which occurred between 2 and 3 hours after the release of 5-hour tourniquets in experimental animals and 2 hours after a 5-hour period of anesthesia for the control animals. Broken horizontal bars indicate the average change for each group.

conducted with both gross and differential counts being made at the same time to obtain the actual values of the cells present. The gross counts were discontinued in subsequent experiments after the trend had been established. Another group of 33 rats was used for differential counts alone. To establish the normal count, one smear was made just after the tourniquets were applied, and other smears were made every hour after the removal of the tourniquets for 3 hours if the animal lived. A third group of 11 rats was injected with dibenamine hydrochloride,* an adrenolytic drug, just before the tourniquets were applied. The procedure, otherwise, was the same as described above. In a fourth group of 9 animals, smears were made from blood taken from the toe at the end of the 5 hour period, just before the removal of the tourniquet. The procedure, otherwise, was the same as described for the second group.

In the present series of shocked rats, an average of 4 counts were made on 144 animals. For each differential count at least 100 cells were counted while in the second group of 33 animals used for differential counts alone and in the control group at least 200 cells were counted. This procedure would appear to establish the validity of the date presented.

Results. The significant feature of the studies reported is a comparison between the blood counts of fatal and non-fatal cases of shock. Undoubtedly, the procedures employed, anesthesia, tourniquet trauma, and handling, of themselves will produce some changes in the white blood cell count. However, the decrease in lymphocyte count found in animals that died is significantly greater than variations in the blood count of normal rats. This can be seen in Fig. 1. There are two obvious factors which *per se* may bring about the degree of scatter seen in Fig. 1. First, it should be emphasized that the onset of profound shock varies in the experimental animals and that death occurs anywhere from 1 to 10 hours after release of the tourniquets. Similarly, the greatest lymphocyte decrease is not always 2 to 3 hours after tourniquet release. However, in order to permit a direct comparison between the different animals, the blood samples recorded here were all taken between 2 to 3 hours after tourniquet removal. This factor may be the cause of some of the variability encountered, as illustrated in Fig. 1. Secondly, the animals begin to revive soon after the anesthesia wears off and the stress resulting from continued attempts to escape from the animal-boards may cause a greater temporary decrease in lymphocytes than would be seen if the animals were free. In five rats subjected to pentobarbital anesthesia for a period of 4 hours only a slight decrease in lymphocyte count from 5 to 11%, an average of 9%, was encountered. The average decrease in lymphocytes in controls carried for 2 hours past a 5-hour period of anesthesia (comparable to a 5-hour tourniquet period) was 12.9%. In contrast, the average decrease in fatal cases of shock was 18.5%.

Group 1. Gross and Differential Counts in Tourniquet Shock. In preliminary experiments on 12 rats, all of the animals showed a decrease in lymphocytes after removal of the

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* Supplied by Dr. W. Gump, Givaudan-Delawanna, Inc., Delawanna, N. J.

TABLE I.
Percentage of Lymphocytes and Time of Death in Dibenamine Treated Rats 2 Hours after the Release of Hind-Limb Tourniquets.

No.	Normal % of lymphocytes	Lymphocytes 2 hr after tourniquet release, %	Time of death after tourniquet release, hr
1	75	37	3
2	78	61	2
3	66	26	3
4	74	47	3
5	75	57	2
6	64	44	2
7	76	58	2
8	78	49	2
9	66	46	3
10	73	52	2
11	67	46	2

TABLE II.
Percentage of Lymphocytes in the Leg Prior to Tourniquet Release and 2 Hours after Release.

No.	Normal % of lymphocytes	Lymphocytes in leg before tourniquet release, %	Lymphocytes in blood 2 hr after tourniquet release, %
1	71	75	20
2	65	84	29
3	60	88	54
4	60	56	45
5	94	86	66
6	73	84	53
7	82	92	82
8	79	90	54
9	90	87	50

tourniquets, irrespective of whether the gross count increased or decreased. In all of these cases the *actual* number of lymphocytes was decreased. Six of the rats showed a decrease in gross count following the tourniquet removal. In these the actual number of lymphocytes decreased more than the actual number of polymorphonuclear neutrophils were increased. For example, the normal count of one animal showed a total of 7700 cells, of which 79% (6083) were lymphocytes and 21% (1617) were polymorphonuclear neutrophils. Two hours after tourniquet removal, the gross count was 5800 with 54% (3132 cells) lymphocytes and 46% (2668 cells) neutrophils. Therefore, the decrease in lymphocytes was 2951 cells and the increase in neutrophils was 1051 cells. A similar relationship was found in all instances where there were decreases in gross counts. On the other hand, there was an increase in the gross count in 6 of the rats. In these, the actual increase in the number of neutrophils was on the average greater than was the decrease in the number of lymphocytes. For example, one animal showed a normal gross count of 6300 cells, of which 90% or 5670 were lymphocytes and 10% or 630 were neutrophils. Two hours after tourniquet removal, the gross count was 6500 cells with 50% or 3250 lymphocytes and 50% or 3250 neutrophils. Therefore, the actual decrease in lymphocytes was 2420 cells while the actual increase in neutrophils was 2620, or 200 cells more.

Group 2. Differential Counts in Tourniquet Shock. In a group of 33 rats, 11 died within 4 hours following removal of the tourniquets, 4 died within 6 hours, one died at 21 hours, and 4 died at some undetermined time during the night. Thirteen of the animals lived. The differential counts of the 20 that died showed a decrease in the percent of lymphocytes, the greatest decrease being 46%, the least decrease 4%, and the average 18.8%. Of the 13 animals that lived, the greatest lymphocyte decrease was 27%, the least decrease 5%, and the average 13.3% (see Fig. 1).

Group 3. Dibenamine Treated Animals. In the group of 11 rats injected intraperitoneally with 2 mg dibenamine hydrochloride all of the anmals died within 3 hours. These animals showed a considerably shorter period of life following removal of the tourniquet and they also showed a greater lymphocyte decrease than untreated rats (Table I). The greatest lymphocyte decrease was 40%, the least 17%, and the average decrease was 24.4%.

Group 4. Differential Counts on Blood in Leg Prior to Release of Tourniquets. In order to determine whether the decrease in lymphocyte count was due to a loss of cells into the traumatized limb, smears were done in 9 rats with blood taken below the tourniquet, just before its removal. These showed an average increase of 11% in lymphocytes. In contrast the decrease in lymphocyte count averaged 24% in the blood taken from the tail vein following the release of the leg tourniquets and the development of shock which resulted in death for all of the animals (Table II).

Discussion. Mann[4] has reported a general increase in the number of leucocytes in the initial stages of shock followed by a leucopenia as the animal approaches death. The magnitude of the change which occurred depended on the stage and intensity of the condition.

The group of preliminary experimental rats reported here showed different stages of shock. In the present experiments the initial changes in gross leucocyte counts were inconstant whereas a leucopenia invariably developed within 2 hours, consisting for the most part of a decrease in the percent of lymphocytes. The fact that, as the gross count decreased, the actual number of lymphocytes decreased more than the neutrophils increased, would seem to indicate a definite effect on the individual types of cells. During this general decrease, it would have been less significant, if the lymphocytes and neutrophils decreased proportionately, maintaining the original ratio.

The lymphocyte decrease would appear to be a result of a reduction in the actual number of cells, caused by decreased production or increased destruction. It has been suggested that lymphocytes may furnish antibodies or inactivate toxins for the body's defense.[5] Dougherty and White[3] have shown that an increase in pituitary-adrenal cortical secretion is paralleled by an increase in lymphocytic dissolution accompanied by an increase in antibody titer in the blood of immunized animals. Selye[6] has also shown that the pituitary-adrenal system is stimulated during the development of shock. The possibility, therefore, exists that this factor may be related to the decrease in the number of lymphocytes.

Munro and Noble[7] found that normal rats, exposed to trauma in the Noble-Collip drum showed a marked fall in lymphocytes. The decrease in number was directly related to the amount of trauma. When the trauma was great enough to cause death the lympholysis was both rapid and severe.

It is possible that the vaso-depressor substance (VDM) described by Shorr, Zweifach and Furchgott[8] as being liberated during the course of shock may be related to the change in lymphocyte count which occurs when the tourniquets have been released.

The early deaths observed in the animals injected with dibenamine hydrochloride may be due to the fact that the compensatory mechanism for combatting the presence of hypotension is made less effective by the action of this drug on the sympathetic nervous system. The secondary changes in the blood picture are similarly enhanced.

Summary. 1. A lymphocyte decrease

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[4] Mann, F. C., *Bull. Johns Hopkins Hosp.*, 1914, **25**, 203.

[5] Best, C. H., and Taylor, N. B., *The Physiological Basis of Medical Practice*, 1945, The Williams and Wilkins Co., Baltimore, Md.

[6] Selye, H., *J. Clin. Endocrinol.*, 1946, **6**, 117.

[7] Munro, D. D., and Noble, R. L., *Fed. Proc.*, 1947, **6**, 168.

[8] Shorr, E., Zweifach, B. W., and Furchgott, R. F., *Science*, 1945, **102**, 489.

occurs within 2 hours following the removal of the tourniquet in fatal shock induced by leg tourniquets in albino rats. 2. There is no decrease in the percent of lymphocytes in blood smears made below the tourniquet at the end of a 5-hour period. 3. Dibenamine hydrochloride increases the onset of death in tourniquet-shock and increases the lymphocyte decline.

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17227. Protecting Effect of Heparin on the Inactivation of Thrombin by Heat.*

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Thrombin and thrombokinase, unlike prothrombin, are oxidizable and become inactive in their oxidized form, as reported previously.[1] Further investigations proved that γ-quantities of heparin inhibit completely the inactivation of thrombin by oxidation. This property of heparin, unknown till now, showed itself to be specific; it can be concluded, therefore, that heparin possesses not only an antagonistic action on thrombin, but, under certain circumstances, has also qualities protecting thrombin.[2] The inactivation of the coagulation factors by means of oxidation supports our previous theory on the role of oxidation in blood coagulation. Our experiments show that increased oxidation diminishes coagulability of blood, whereas oxygen deficiency accelerates coagulation. Therefore breathing itself must be the essential regulator in blood coagulation *in vivo* as has been shown.[3] The thrombin-protecting effect of heparin, which contrasts with its inhibitory effect on coagulation, is not clarified in our earlier work. It is supposed that this question could be answered by clearing up the nature of the plasma factor (co-factor) of heparin. This supposition is supported by the fact that the unknown plasma factor can modify the heparin effect, as may be seen by the different reactions of heparin against plasma and purified fibrinogen. Heparin has no coagulation-inhibitory effect on purified fibrinogen.[4]

In the present work we investigated whether heparin has an inhibiting effect on heat inactivation of thrombin, similar to its protecting effect toward oxidation. We used for our investigations thrombin and heparin (Liquemin, Hoffmann-La Roche). The activity of thrombin was measured with a solution of fibrinogen, containing 15 mg/ml of dry substance; the time of coagulation was noted in seconds. The solutions used for heat inactivation contained thrombin mixed with varied quantities of heparin. In each experiment the mixtures of thrombin and heparin were kept exactly 10 minutes in the water bath. A control series was similarly treated without heparin. The activity of thrombin was measured 1 hour after the samples were removed from the water bath.

It can be seen from Table I that the inactivation of thrombin by heat is inhibited most efficiently by an equal quantity of heparin. Smaller amounts of heparin inhibit less; however, a larger amount does not essentially increase the inhibitory effect. The heat inactivation of thrombin in the presence of an equal amount of heparin is demonstrated in a curve which is essentially similar to the inactivation curve of thrombin following oxidation.

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* Aided by the Roche Research Fund, Basel.

[1] Palos, L. A., *Nature*, in print.

[2] Palos, L. A., *Experientia*, 1949, **5**, 207.

[3] Palos, L. A., *Acta Medica Scandinavica*, in print.

[4] Howell, W. H., and Holt, E., *Am. J. Physiol.*, 1918-19, **47**, 328.

TABLE I.
Activity of Thrombin after Keeping 10 Min. at Different Temperatures in Water Bath with and without Heparin.

Temp. °C	Thrombin: Heparin: pro ml dest water	1 mg 1.5 mg	1 mg 1 mg	1 mg 0.5 mg	1 mg 0 mg
20		15″	15″	16″	16″
30		16″	16″	20″	20″
40		17″	18″	22″	29″
50		23″	24″	34″	50″
60		25″	26″	51″	500″
70		28″	29″	66″	1200″
80		34″	36″	88″	∞
90		44″	45″	90″	∞
100		51″	52″	260″	∞

Without heparin inactivation of thrombin is strongly accelerated at 50°C; between 70 and 80°C it loses its activity, *i.e.*, the fibrinogen cannot be further coagulated. In contrast, a solution of thrombin containing a corresponding amount of heparin, after even 10 min. boiling (100°C), causes a strong coagulation of fibrinogen; the activity diminishes only to one-third of the initial value.

The protecting effect of heparin shows differences concerning oxidation of thrombin and inactivation by heat. In the first case γ-quantities of heparin were enough to stop oxidation completely. For protecting the inactivation by heat there are required at least equal quantities of heparin to produce a marked effect. Total inhibition of heat inactivation could not be obtained. If the treatment with heat is prolonged, the thrombin loses its activity despite the protecting effect of heparin, but much more slowly than without heparin.

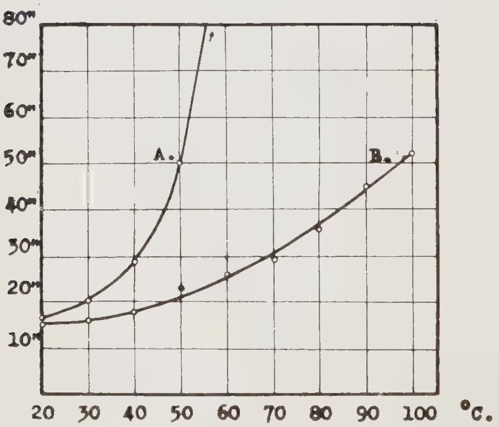

FIG. 1.
Activity of thrombin after keeping 10 minutes at different temperatures in water bath without (A) and with (B) heparin.

A similar effect to that of heparin could not be produced with other, not esterified di- and polysaccharides, neither in the oxidation experiments nor in the investigation on heat inactivation.

Synthetic polysaccharidesulfonates, which correspond to heparin in inhibition of coagulation, were not investigated.

These new facts, the total inhibition of oxidation and the high grade of inhibition of inactivation of the thrombin by heparin may lead to a new conception of the relationship between thrombin and heparin.

Summary. The inactivation of thrombin by heat is sharply inhibited, if it is mixed with equal parts of heparin. Heating a mixture of thrombin-heparin (1:1) for 10 minutes to 100°C causes only a slight decrease of activity.

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17228. Relationship Between Spontaneous Activity and Metabolic Rate as Influenced by Certain Sympathomimetic Compounds.

F. A. Waterman. (Introduced by A. C. Kuyper.)

From the Department of Biology, Wayne University, Detroit, Mich.

The measurement of spontaneous activity in small animals appears to be dependent upon the method used, many of which have been described in the literature. Results reported by the author,[1] based upon change of resistance in an electrical circuit when an electrode is moved in and out of a salt solution, differ from those obtained by others. Schulte *et al.*,[2] using a work adder type of kinisimeter, reported that d-N-methyl-α-methyl-β-phenylethylamine HCl (desoxyephedrine) was a more powerful stimulant than dl-benzedrine HCl. They also found that the l-isomer of N-methyl-β-cyclohexylisopropylamine HCl had a greater stimulating effect than the d-isomer. The writer was unable to confirm either of these results. The results obtained with all kinisimeters may be criticized on the basis that they do not record the very small movements produced by compounds, such as desoxyephedrine which causes the animal to swing his head continuously in a rotary manner. Since oxygen is consumed in making these movements, it was considered that a measurement of oxygen consumption and determination of the metabolic rate would be of use in determining spontaneous activity effects.

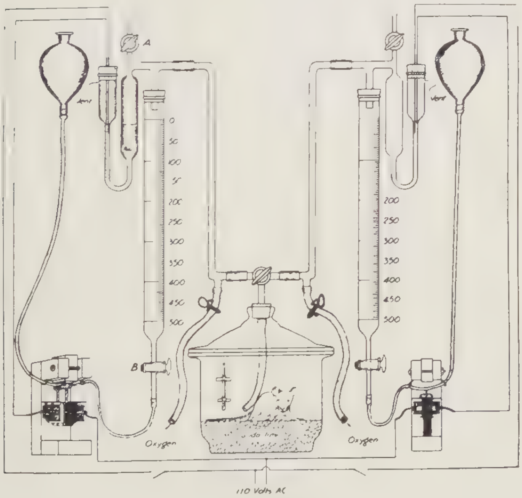

Fig. 1.

Apparatus for the continuous estimation of the metabolic rate of small animals.

Apparatus. The types of apparatus used for oxygen consumption determination have been reviewed by Tabor and Rosenthal.[3] The apparatus used in this study is shown in Fig. 1. The tops of two 500 ml dispensing burettes were connected to a large pyrex desiccator. A thermometer was fastened to the inside wall of the desiccator in order that the vapor tension could be corrected. The upper ends of the burettes were connected to specially constructed manometers filled with a saturated solution of sodium chloride containing a small amount of aerosol to decrease surface tension. Two expansion chambers are included in each of the manometers to prevent the electrolyte from being accidentally drawn into the burettes or spilled to the outside when the apparatus is being filled with oxygen. It will be noted that a small vent is blown in the side wall of the expansion chamber containing the electrodes. These electrodes control the current passing through Allen and Bradley solenoids, which, in turn, control the flow of water from the 500 ml leveling bulbs into the burettes. A simple switch is included in each solenoid circuit in order that the current may be broken at will, to permit filling the burettes with oxygen. The base of the desiccator is filled with soda lime and a disc of heavy screen wire (hardware cloth) replaces the desiccator plate.

All measurements are made in a constant temperature room. The animal is placed in the chamber and the apparatus washed out several times with oxygen. As the animal consumes oxygen the side arm of the manometer falls and breaks the electrical circuit, causing the solenoid to drop and allowing water to enter the burette. When the water has replaced the consumed oxygen, the original level of the electrolyte in the manometer is restored, automatically closing the circuit and the solenoid again pinches the rubber tube which stops the flow of water. When one burette is depleted of oxygen, the 3-way stopcock is turned and the second burette is thrown into service while the first one is being refilled. Thus the oxygen consumed can be determined continuously.

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[1] Waterman, F. A., *Science,* 1947, **106**, 499.

[2] Schulte, J. W., Reig, E. C., Bacher, J. A., Jr., Lawrence, W. C., and Tainter, M. L., *J. Pharm. Exp. Therap.,* 1941, **71**, 62.

[3] Tabor and Rosenthal, *Am. J. Physiol.,* 1947, **149**, 449.

In the calculations an R.Q. of 0.725 was assumed.[4] The metabolic rate in this investigation is defined as calories of heat per hour per gram of body weight. All measurements were made with animal in the post absorptive state. This method is particularly useful for the rapid screening of drugs. The accuracy of such methods as that of Haldane[6] and accurate surface area determinations are unnecessary.

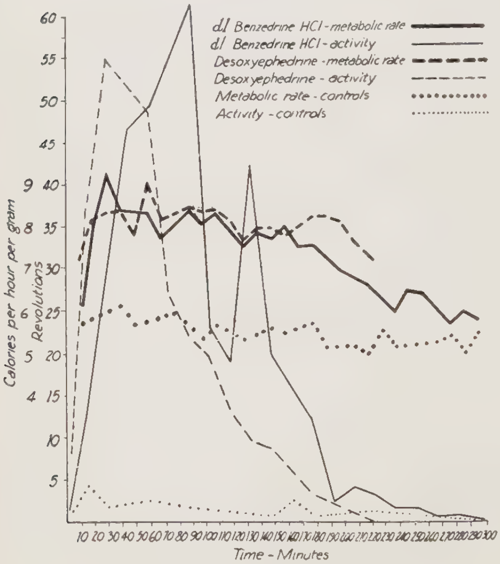

FIG. 2.

Graph showing the effect of dl-benzedrine and desoxyephedrine on the metabolic rate and central nervous excitation.

Experimental. Control animals were injected with normal saline solution and their metabolic rate determined at 10-minute intervals over a period of 6 hours. The experimental animals were injected with the compound being studied and determinations of oxygen consumption in 10-minute intervals were begun at once. The control and experimental data obtained were plotted along with the data previously obtained from activity studies[1] in order that the relationship between metabolic rate and activity could be readily seen. The following compounds were studied:

	No. of rats	Dosage (mg/kg)
d-N-methyl-α-methyl-β-phenylethylamine HCl (desoxyephedrine)	6	5
dl-benzedrine HCl	6	5
1(3′,4′-dihydroxyphenyl)-2-isopropylaminoethanol HCl (Isuprel)	6	1
N-methyl-β-cyclohexylisopropylamine HCl		
d-isomer	6	5
l-isomer	6	5
Neosynephrine HCl	6	0.07

Fig. 2 shows the effects of both benzedrine and desoxyephedrine (d-N-methyl-α-methyl-β-phenylethylamine HCl) on the metabolic rate and activity of the animals. The maximum activity as well as the maximum metabolic rate are higher in the case of benzedrine

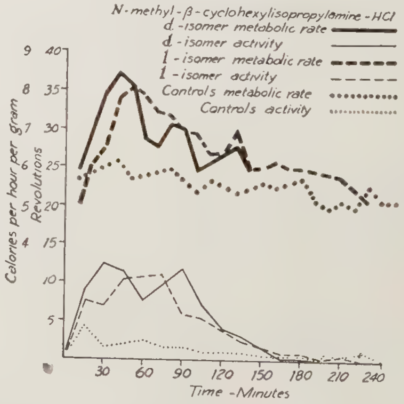

FIG. 3.

The relationship between the d- and l-isomers of N-methyl-cyclohexylisopropylamine HCl as regards their effect on metabolic rate and central nervous excitation.

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[4] Griffith and Farris, 1942, The Rat in Laboratory Investigation, p. 186.

[5] Diack, Samuel L., *J. Nutrition*, 1930, **3**, 289.

[6] Haldane, J. B. S., *J. Physiol.*, 1892, **13**, 419.

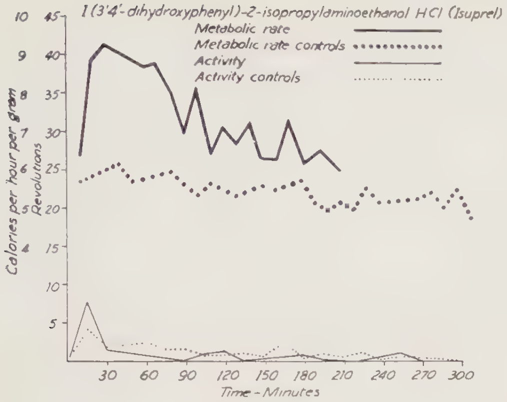

FIG. 4.

The effect of Isuprel on the metabolic rate and central nervous excitation.

than in the case of desoxyephedrine.

The effects produced by the d- and l-isomers of N-methyl-β-cyclohexylisopropylamine HCl are shown in Fig. 3. The d-isomer is more active than the l-isomer as shown by effects on both activity and the metabolic rate. All of the above-mentioned compounds show parallel increases in both activity and metabolic rate.

The injection of 1(3′,4′-dihydroxyphenyl)-2-isopropylaminoethanol HCl (Isuprel), Fig. 4, produces, if anything, a slight decrease in the activity of the normal animal. However, the metabolic rate is markedly increased, being even higher than that produced by dl-benzedrine. The animal is in a state of complete collapse and breathing rapidly. His heart rate is greatly increased, the peripheral vessels are greatly dilated and the temperature is elevated. The effect on the metabolic rate is clearly not due to movements produced by central nervous stimulation. This compound has been shown to have a relaxing effect on bronchiole spasm induced by pilocarpine and to markedly decrease the blood pressure.[7] The increased oxygen consumption is attributable in part to increased heart rate, respiratory movements, and possibly to increased tissue oxidation.

Summary. Comparisons were made between the oxygen consumption and increased movements induced by the central nervous excitatory effects of a number of physiologically active organic compounds. After injection of several of these compounds, benzedrine, desoxyephedrine and N-methyl-β-cyclohexylisopropylamine HCl, oxygen consumption and voluntary movements varied in direct relationship to each other. After the injection of another compound (Isuprel), oxygen consumption increased greatly, but there was a decrease in activity. Oxygen consumption can be used as a measure of voluntary muscular activity only under limited conditions.

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[7] Lands, A. M., personal communication.

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17229. A Simplified Tissue Culture Technic.

H. GROSSFELD.* (Introduced by J. B. Murphy.)

In a single chicken embryo, we find all that is required for preparing tissue cultures: the ingredients of the culture medium as well as the explant. Salt-glucose mixtures are replaced by amniotic fluid from the same embryo from which the tissues are obtained. An egg shell sector about 1¼ inches in diameter is cut out of the large egg pole on the 8-9th day of incubation, and the contents are poured cautiously into a Petri dish so as to avoid bursting the very delicate amniotic membrane. The membrane is then punched with a thin, sharp cannula and 2-3 cc of amniotic fluid are aspirated. The shell sector should not be larger than indicated, otherwise the amniotic membrane may be easily damaged. Only completely transparent fluid should be used; turbid fluids are mixtures of yolk and amniotic fluid and will inhibit growth. Tissue for explants is then cut out and the remaining embryonic tissue provides the embryonic juice. The culture medium consists of embryonic juice and amniotic fluid 1:1. The excess of the amniotic fluid may be used for all purposes where saline or other salt mixtures are usually used, *e.g.*, washing of tissue, etc. The growth of cultures in this medium is not inferior to the growth of cultures in other fluid media. The cells are as normal in appearance as cells from a plasma culture (Fig. 1). Amniotic fluid not only replaces but also, in many regards, surpasses Tyrode's and other salt-glucose solutions in that it does not require sterilizing, filtering, or pH-adjusting. This liquid medium method may be recommended for cultures where the primary medium is to be removed for experimental purposes and replaced by other media. It is also convenient for obtaining growth of single cell layers for investigation in dark-field illumination, in phase contrast microscopy and in electron microscopy.

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* Fellow of the World's Health Organization.

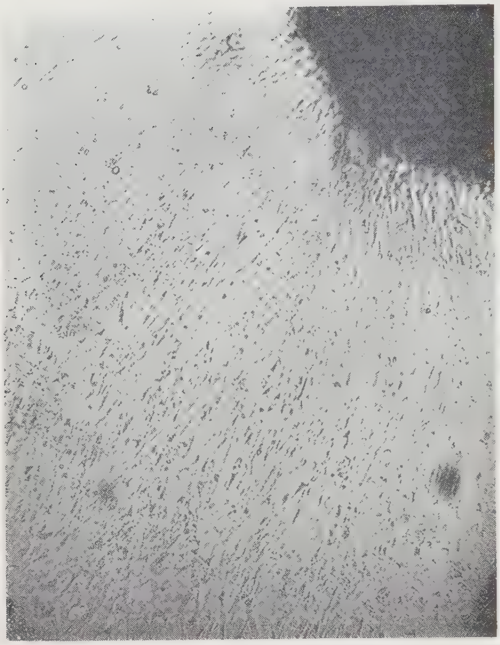

FIG. 1.
Photomicrograph of a living culture of chick embryo heart tissue cultured in embryonic juice and + amniotic fluid (1:1), all obtained from the same embryo.

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17230. A Method of Obtaining Influenza Virus Growth Curves in Individual Eggs.*

ROBERT H. GREEN AND MOYE W. FREYMANN.† (Introduced by Francis G. Blake.)

From Department of Internal Medicine, Yale University School of Medicine, New Haven, Conn.

Growth curves of influenza virus propagated in the allantoic sac of embryonated eggs have been described by several investigators.[1-6] However, most of the studies reported have been based on results obtained with the pooled allantoic fluids of groups of eggs sacrificed at various intervals of time following inoculation of virus. Relatively little data concerning the characteristics of such curves in individual eggs are available. The use of pooled fluids has yielded valuable information as to the average rate and extent of virus multiplication and, with the aid of special technics, the step-like nature of the curves.[4-6] Growth curves in individual eggs should provide similar information, in addition to presenting a continuous picture of the growth curve in each egg. Recently Hoyle[5] has described growth curves of influenza A virus

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* Aided by a grant from the United States Public Health Service.

† James Hudson Brown Memorial Research Fellow.

[1] Miller, G. L., *J. Exp. Med.*, 1944, **79**, 173.

[2] Henle, W., and Henle, G., *Am. J. Med. Sci.*, 1944, **207**, 705.

[3] Ziegler, J. E., Jr., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1944, **79**, 361.

[4] Henle, W., Henle, G., and Rosenberg, E. B., *J. Exp. Med.*, 1947, **86**, 423.

[5] Hoyle, L., *Brit. J. Exp. Path.*, 1948, **29**, 390.

[6] Henle, W., and Rosenberg, E. B., *J. Exp. Med.*, 1949, **89**, 279.

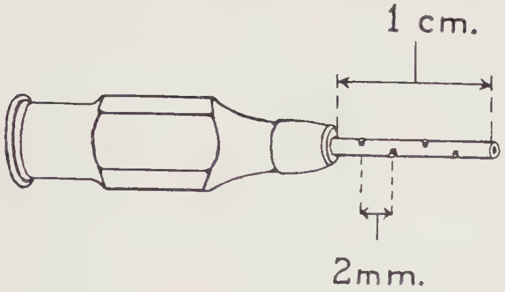

FIG. 1.
Needle devised for aspirating allantoic fluid.

in individual eggs from which allantoic fluid was removed at hourly intervals. However, no details of the technic used for sampling were given by him. The present paper describes a method by which individual growth curves may be obtained.

Materials and methods. Samples of allantoic fluid were aspirated through needles especially devised for this purpose. Aspirating needles were made from ordinary 20 gauge "hypodermic" needles by cutting off the ends and filing 4 holes in the sides of the short lengths of needle remaining. As illustrated in Fig. 1, the length of the needle, from attachment to the hub to the tip, is one centimeter. The holes along the shaft are spaced alternately on opposite sides, 2 mm apart, the first hole being 2 mm from the tip and the fourth 2 mm from the hub. A needle of this type was made readily, in 5 to 10 minutes, with the aid of the flat metal file commonly used to remove the tips of glass ampoules. However, the filing could be done more efficiently by means of a dental drill fitted with a flat cutting disc. During the process of filing, the needle was held firmly by attaching it to a small syringe equipped with a "Luer-Lok" device, and a stylet was inserted before making the side holes.

Needles somewhat similar to those described above have been used previously, especially for flushing the allantoic sac,[7] and for collecting large amounts of allantoic fluid.[8]

Preparatory to inoculation of virus, each egg was candled and a mark was made on the side of the shell in the vicinity of the embryo, directly over an area of chorio-allantoic membrane where no large blood vessels were seen. A hole of about the same size as a cross section of the needle was then drilled through the shell at the designated mark. The hole used for inoculation of virus, as a rule, was used for subsequent aspirations. After inoculation and after each aspiration the holes were sealed with a drop of paraffin-bee's wax mixture. Prior to aspiration the eggs were candled to determine viability; the wax seals were removed with a hot metal spatula and the areas about the holes cleansed with alcohol. After attachment to a syringe of suitable capacity the needle was slowly inserted to the hilt and suction applied by withdrawing the plunger. At each aspiration exactly 0.2 cc of allantoic fluid was withdrawn and added to 0.8 cc of 0.85% NaCl. Serial two-fold dilutions were then made in saline in 0.5 cc amounts. To each tube 0.25 cc of 1% chicken RBC was added and readings were made after the cells had settled out, on standing at room temperature for about an hour. The end-point was taken as the highest dilution of allantoic fluid in which a pattern characteristic of complete or ++++ agglutination occurred. The PR8 strain of influenza A virus was used exclusively.

Experimental. Groups of 10 to 20 embryonated hens' eggs, on the tenth day of incubation, were inoculated with influenza virus via the allantoic sac. As a rule, the inoculum consisted of approximately 100 ID_{50} in a volume of 0.05 cc. The eggs were subsequently incubated at 37°C. Chilling was not done, and the eggs were removed from the incubator only for the brief periods of time required for each aspiration. When small inocula of virus were used, the first aspiration was done at 18 hours after inoculation because preliminary experiments had shown that hemagglutinins usually could not be demonstrated before that time. Repeated aspirations were then done at arbitrarily chosen intervals as long as the embryos survived.

In Fig. 2 are shown the curves, representing

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[7] Henle, W., and Henle, G., *Am. J. Med. Sci.*, 1944, **207**, 717.

[8] Taylor, A. R., Sharp, D. G., McLean, I. W., Jr., Beard, D., and Beard, J. W., *J. Immunol.*, 1945, **50**, 291.

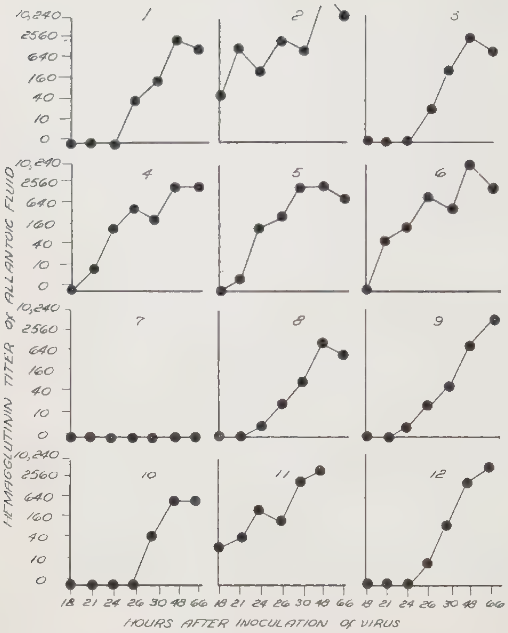

Fig. 2.

Growth curves, based on hemagglutination titers, of the PR 8 strain of influenza virus in individual eggs. Results are those obtained in a single experiment, in which each of 12 eggs received an inoculum of approximately 100 ID_{50} of virus.

the serial hemagglutination titers in individual eggs, obtained in an experiment in which each of a group of 12 eggs received a standard inoculum of about 100 ID_{50} of virus. While some of the curves are quite similar, others show obvious differences with respect to time of appearance of detectable hemagglutinin, slope of curve and maximum titer of hemagglutinin. Moreover, as previously demonstrated by Henle[4,6] and Hoyle,[5] several of the eggs, especially numbers 2, 4, 6 and 11, show step-like increases in the amount of virus. The fact that egg number 7 never became positive may have been due to failure to inoculate it; however, in other similar experiments, an occasional egg has shown an even longer lag phase, hemagglutinins first making their appearance some 90 hours or more after inoculation. All embryos in this experiment were alive 48 hours after inoculation of virus but when candled just prior to the 66 hour aspiration all except number 10 were found to be dead. In most similar experiments a rather high mortality has occurred somewhat earlier.

Comment. The method described is technically easy; relatively small numbers of eggs are required, and the growth curves obtained are individual curves rather than those based on group sampling. Furthermore, this method provides a suitable way of studying the differences between curves in untreated eggs and those in eggs treated with various substances which inhibit the multiplication of influenza virus. However, a high mortality rate usually occurs and about 50% of the eggs die before satisfactory growth curves are obtained. Mortality varies considerably from one experiment to another and apparently depends upon a number of factors which have not been investigated fully. Only a small percentage of the eggs become contaminated with bacteria. In most instances aspiration yields clear allantoic fluid, but occasionally bloody fluid or yolk is obtained. A higher mortality and greater difficulty in aspiration are most likely to be encountered with eggs which have been incubated for only 10 days or less.

Summary. With the aid of an easily constructed needle a method has been devised for obtaining growth curves of influenza virus in individual embryonated eggs.

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17231. Some Effects of Dilution on the Nutritive Value of Dialyzed Plasma and Embryo Juice.*

PHILIP R. WHITE AND ETIENNE LASFARGUES. (Introduced by S. P. Reimann.)

From the Lankenau Hospital Research Institute and the Institute for Cancer Research, Philadelphia, Pa.

The classic substratum for tissue cultures, developed by Harrison,[1] Burrows,[2] and Carrel,[3] consists of chick plasma clotted with chick embryo juice and with an overlay of embryo juice variously diluted. Such a plasma clot without embryo juice overlay is insufficient to support prolonged tissue growth except when frequently renewed by transplanting the cultures to fresh substratum. It supplies an ill-defined but far from negligible level of nutritional factors. In any precise study of cell nutrition, these factors must either be clearly defined or, if possible, eliminated before the substratum can be satisfactorily evaluated as a base line. Various attempts have been made to arrive at such an evaluable base line. Porter and Hawn[4] have used clots prepared from purified fibrinogen coagulated with thrombin. Both these substances are more clearly defined and more nearly inert than are plasma and embryo juice, but they are still not completely defined. Evans and Earle[5] have been successful in developing a procedure for cultivation of tissue cultures on sheets of perforated cellophane, which is nutritionally fully inert. This is a very satisfactory solution of the nutritional problem, but such cellophane is not easily available to all workers throughout the world. Lewis,[6] White,[7] and others have grown cultures directly on glass. This is likewise a fully satisfactory solution of the nutritional problem but the results obtained are somewhat unreliable.

Fischer[8] has approached the problem from quite a different angle by seeking to eliminate the nutritional factors from the classic plasma clot. He showed that when chick plasma and embryo juice, after thorough dialysis against a dextrose-Ringer's solution, were used in the preparation of clots on which to cultivate chick fibroblasts, no growth whatever would occur thereon. He concluded that such a clot was nutritionally inert and was not attacked by the tissue enzymes. This being true, it could safely be used as an inert substratum against which to study the nutritive properties of various supernatants. Dialyzed plasma and dialyzed embryo juice have been rather extensively used in such studies.[9]

Fischer states[8] that implanted tissues grew less and disintegrated more quickly on dialyzed plasma than they did in Tyrode's solution alone. If the defect is merely one of nutrient deficiency, it is difficult to see how Tyrode's solution could be the less deficient of the two. The alternative possibility exists that the defect may be due not merely to a low level of nutritive value but to some more positive injurious effect. Indeed, Fischer himself speaks of "den Eindruck einer direckten toxischen Einwirkung."[8] For this and other reasons we decided to repeat Fischer's work. Preliminary experiments were carried out by somewhat simplified procedures, using hanging drop cultures in place of Carrel flasks and chick heart fibroblasts in place of frontal bone fibroblasts.

Fischer used an undiluted dialyzed embryo

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* Aided by a grant from the American Cancer Society, recommended by the Committee on Growth of the National Research Council.

[1] Harrison, R. G., PROC. SOC. EXP. BIOL. AND MED., 1907, **4**, 140.

[2] Burrows, M. T., *J. Am. Med. Assn.*, 1910, **55**, 2057.

[3] Carrel, A., *J. Exp. Med.*, 1913, **17**, 14.

[4] Porter, K. R., and Hawn, C. v. Z., PROC. SOC. EXP. BIOL. AND MED., 1947, **65**, 309.

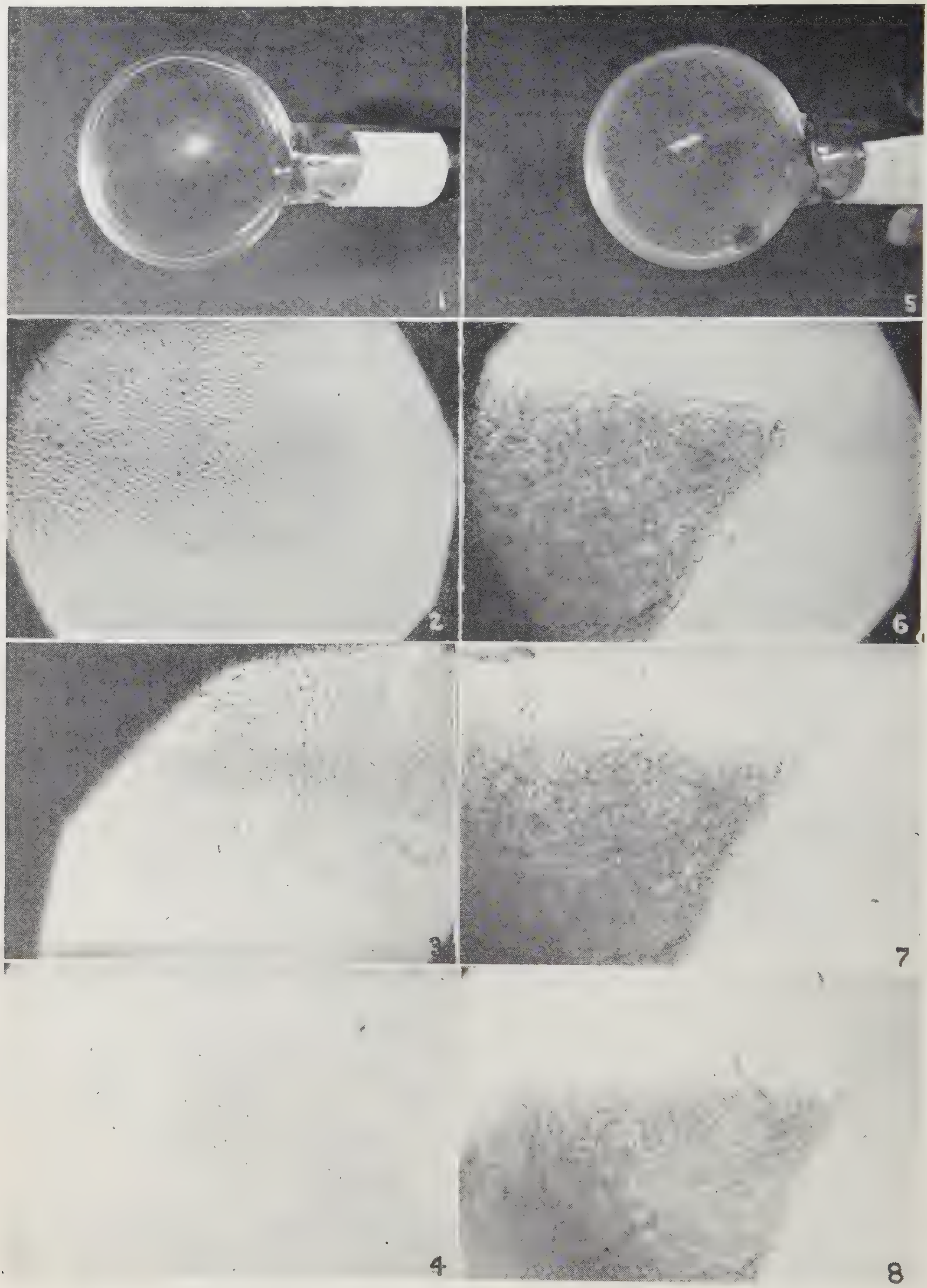
[5] Evans, V. J., and Earle, W. R., *J. Nat. Cancer Inst.*, 1947, **8**, 103.

[6] Lewis, Warren H., *Carnegie Inst. Wash., Contrib. to Embryology*, 1926, **18**, 1.

[7] White, P. R., *Growth*, 1947, **10**, 231.

[8] Fischer, A., *Acta Physiologica Scandinavica*, 1941, **2**, 143.

[9] Fischer, A., Astrup, T., Ehrensvard, G., and Oelenschlager, V., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 40.



(All photographs are of living material, made with 16 mm panatomic film. Fig. 1, 5, 9, and 13 (Carrel flasks) are × 1. All others are about × 76).

FIG. 1. Twenty-four hour culture of chick frontal bone fibroblasts on the control medium consisting of chick plasma clot with overlay of chick embryo juice diluted 1:9 with Tyrode's solution. The halo of migrating cells is clearly evident.

FIG. 2. Detail from the above culture showing the compact, uniform growth of finely granular cells.

FIG. 3. Margin of the same culture at the end of 4 days. The cells contain many small fat globules and are larger than in Fig. 2, but are in excellent condition.

FIG. 4. Area from about midway between the original implant and the edge of the flask, taken at the end of 10 days. The uniform character of the growth and relatively clear cytoplasm of the cells are evident.

FIG. 5. Twenty-four hour culture of chick frontal bone fibroblasts on an undiluted dialyzed plasma-dialyzed embryo juice clot, with undiluted dialyzed embryo juice overlay. No growth has occurred.

FIG. 6, 7, 8. Details of the above culture after 24 hours, 4 days, and 10 days. There has been no migration or change other than disintegration of the cells within the implanted bit of clot.

juice in the preparation of both his substratum and his experimental supernatant.[8] We have obtained our most satisfactory control results (undialyzed media) with nutrients in which the embryo juice* was diluted about 1 to 9 with Tyrode's solution. In order that our experimental cultures might be more nearly comparable with the controls, we therefore carried out cultures not only with undiluted embryo juice but with dilutions approximating those of our controls. Our experiments with undiluted embryo juice fully confirm Fischer's findings, but, to our surprise, cultures prepared with diluted dialyzed embryo juice grew quite well over the 48-hour periods which can be followed in hanging drops. This suggested that the undiluted material remaining after dialysis was perhaps not *deficient,* that is, lacking in essential nutrient factors, but merely *defective* due to unsatisfactory concentrations or proportions of its constituents. These initial experiments were, however, not duplicates of those reported by Fischer. We therefore carried out another series of cultures, repeating as nearly as possible Fischer's second procedure[8] and extending the study to the use of diluted media.

One of us (E.L.) spent a month in the summer of 1946 in Fischer's laboratory in Copenhagen and was therefore familiar with his methods. In the preparation of plasma and in dialysis of both plasma and embryo juice we followed Fischer's procedures exactly. Twenty-five ml of plasma, from blood drawn by heart puncture from a young cockerel into a heparinized syringe, were transferred to a sterile (autoclaved) cellophane dialysis tube under sterile precautions and were dialyzed for 8 days in the refrigerator at about 4°C against 1.5 liters of Ringer's solution containing 0.1% dextrose. The solution was not renewed.[8] Freshly prepared juice from 9 day chick embryos was similarly treated. All dilutions were made with Tyrode's solution without dextrose. It was found that satisfactory clots could not be obtained with dilutions higher than one part of embryo juice in two parts of Tyrode's solution. A series was set up with 16 Carrel flasks, 4 flasks containing each of the following solutions: 1) control, undiluted plasma (0.5 ml) coagulated with 0.5 ml of embryo juice diluted 1:2, supplied after clotting with a supernatant of embryo juice diluted 1:9; 2) Fischer's cultures, consisting of undiluted dialyzed plasma (0.5 ml) clotted with 0.5 ml of undiluted dialyzed embryo juice, and with a supernatant of undiluted dialyzed embryo juice; 3) similar to the latter, but with 0.5 ml of undiluted dialyzed plasma clotted with dialyzed embryo juice diluted with an equal part of Tyrode's solution (0.5 ml), and a supernatant of dialyzed embryo juice likewise diluted with an equal part of Tyrode's solution; and 4) undiluted dialyzed plasma (0.5 ml) clotted with dialyzed embryo juice diluted with 2 parts of Tyrode's solution (0.5 ml), and a supernatant of dialyzed embryo juice similarly diluted. In each flask was placed a single fragment of a rapidly growing homogeneous culture of fibroblasts derived from the frontal bone of a 14-day chick em-

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* Embryo juice was prepared by finely triturating with sharp scissors without addition of fluid, allowing to stand for one-half hour, and then centrifuging.

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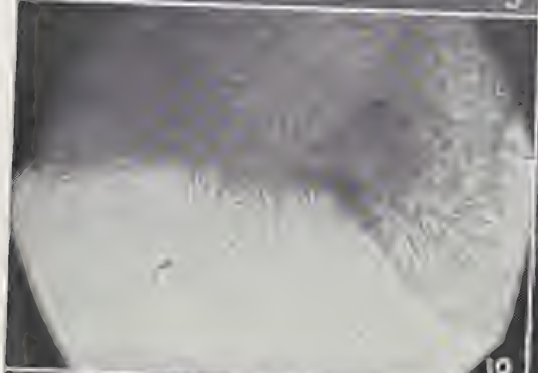
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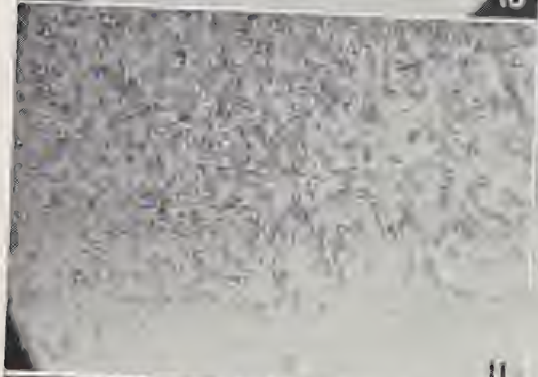
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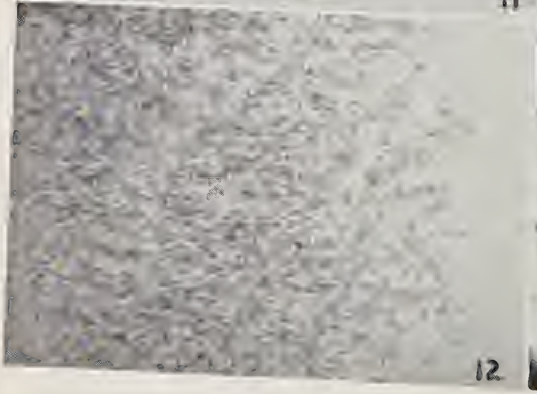
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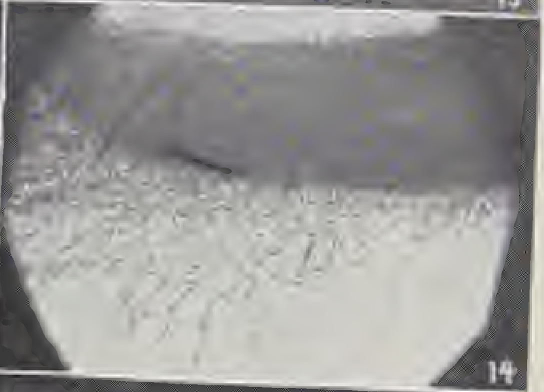
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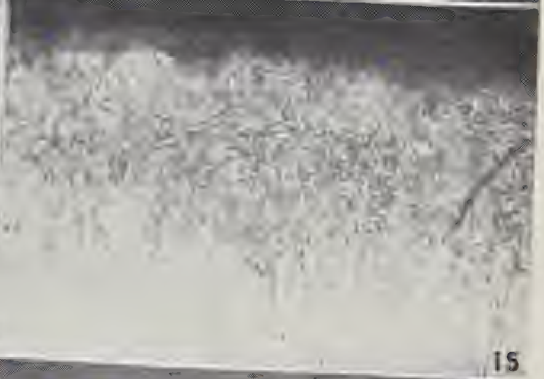
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FIG. 9. Twenty-four hour culture of chick frontal bone fibroblasts on a clot prepared with dialyzed embryo juice diluted 1:1 with a dextrose-free Tyrode's solution, with overlay of this

same dilution. The halo of migrating cells is much smaller than in Fig. 1, but is still quite evident.

FIG. 10. Detail from the above culture showing the sparse but evident migration of fibroblasts.

FIG. 11. The same culture as Fig. 10 at the end of 4 days (compare with Fig. 3). The zone of migrating cells is fairly wide and quite uniform. The cells themselves are large, larger than in Fig. 3, but are packed with fat globules, many of which are very large.

FIG. 12. The same culture after 10 days. Outlines of single cells are no longer visible, disintegration having taken place.

FIG. 13. Twenty-four hour culture of chick frontal bone fibroblasts on a clot prepared with dialyzed embryo juice diluted 1:2 with dextrose-free Tyrode's solution, with overlay of the same dilution. The considerable halo of migrating fibroblasts is evident.

FIG. 14. Detail from the above culture. The area of growth is both qualitatively and quantitatively better than at a 1:1 dilution (Fig. 10).

FIG. 15. The same culture after 4 days. Growth is somewhat more compact than at a 1:1 dilution (Fig. 11) but otherwise not very different.

FIG. 16. The same after 10 days. Cells in the original migrating zone (transverse band) are fatty but in the gap left between this and the original implant by lysis and contraction of the clot, subsequently patched, the new cells have clear-cut outlines and are not excessively filled with fat. This culture is still very much alive and growing, though very slowly.

bryo and maintained on the standard chick plasma-dilute embryo juice medium through four successive passages. Cultures were examined and photographed at the end of 48 hours, 4 days, and 10 days. Nutrient was replaced in each of the flasks after 2, 4, 6 and 8 days.

Results. The results were clear cut, consistent both among themselves and with the results of previous experiments using hanging drop cultures. Growth on the control nutrient was normal and very rapid, so that at the end of 10 days the entire *bottoms* of the flasks were covered. The clot had undergone lysis around the implant and was patched on the 4th day; new growth had covered the patch by the 10th day (Fig. 1-4). On the undiluted dialyzed plasma dialyzed embryo juice medium, in complete agreement with Fischer's results, there had been absolutely no growth and the cells had undergone histolysis (Fig. 5-8). On dialyzed plasma containing dialyzed embryo juice diluted 1:1, however, there had been very considerable growth (migration). At 24 hours this reached a maximum width of about 0.1 mm. At 4 days the band was about 0.8 mm wide, the cells were *larger* than the controls and were packed with fat globules. At 10 days they had grown no further and were mostly dead and broken down (Fig. 9-12). Dialyzed plasma clotted with embryo juice diluted 1:2 did not give firm uniform clots, although they were sufficiently coherent to attach the cultures satisfactorily and to provide a satisfactory substratum on which growth could take place. At 24 hours the growth zone was about 0.5 mm wide, 5 times as great as at 1:1 dilution. At 4 days this had about doubled, that is, was about the same width as in the 1:1 dilution. The growth was, however, denser and the cells somewhat less fatty. At 10 days, some lysis had occurred (patched at 8 days), the band was about 2 mm wide, cells had grown out into the patched areas, and the cells were still alive (Fig. 13-16).

Discussion. The results agree with those of Fischer[8] in showing that an undiluted dialyzed plasma-dialyzed embryo juice clot with undiluted dialyzed embryo juice overlay does not support growth of a homogeneous strain of frontal bone fibroblasts and brings about rapid breakdown of implanted cells. Yet dilution of the overlay with Tyrode's solution lacking dextrose, which is a non-nutritive and purely protective solution, restores the capacity to support at least a residual degree of growth, and the degree of restoration is, within the limits set by the clotting capacity of the medium, proportionate to the degree of dilution. It is evident that the failure of the undiluted dialyzed material to support growth was not due solely to a deficiency, since any deficiency would only have been accentuated by dilution. It must, therefore, have been due to some positive defect. Such positive defects may be of two sorts. There may be present a non-dialyzable growth inhibitor, either a high molecular weight substance or a prosthetic group attached to such a substance, whose effects are minimized by dilution. One point in Fischer's data might lend support to the idea of such a toxic factor. Fischer reports that either egg albumin or insulin, when added to dialyzed plasma, will protect cells placed thereon from histolytic disintegration. He attributes this effect to the nutritive properties of the labile S-S and S-H bonds in these materials, but both egg albumin and insulin are known to be highly active adsorbents capable of detoxifying various media. This appears to be the effect of serum albumin when added to media for the cultivation of bacteria[10] and may have been a factor in Spratt's experiments[11] with explanted early chick blastoderms (not tissue cultures). It seems quite possible that the beneficial effect of egg albumin or insulin when added to dialyzed plasma may be of similar origin. On the other hand, the defect might be due to an imbalance of nutritive materials. The plasma and embryo juice were dialyzed against a Ringer's solution containing 0.1% dextrose but lacking phosphate. The diluent used in preparing media (Tyrode's solution) contained no dextrose but did contain phosphate. The diluent was thus *not* in equilibrium with the dialyzed materials, and would both extract dextrose therefrom and add phosphate thereto. If 0.1% dextrose was too high a concentration for fibroblast growth, the defect would be corrected by dilution. Since phosphate is required for growth, the defect might be a simple deficiency as postulated by Fischer, but not necessarily a deficiency of organic materials. However, Fischer's evidence, that a suitable amino-acid mixture prepared with physiological salt solution rather than Tyrode's solution does, in fact, partially correct the defect of dialyzed plasma-embryo juice, shows that phosphate was not the critical factor. It shows also that the low molecular weight materials removed by dialysis, such as the amino acids, *are* of great and at this level critical importance.

All of this evidence, however, still leaves unresolved the question of the nature of the postulated positive toxic defect in the dialyzed materials. In any case, our observations, though limited, suggest that it is unsafe to assume that dialyzed plasma or embryo juice is truly inert. Until further experiments have been carried out to clarify the effects of dilution on dialyzed extract, we feel that these materials should not be used in the preparation of substrata on which to test the nutritive value of supernatants. We believe that bare glass or cellophane are still the only really safe bases for such studies.

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[10] Davis, B. D., and Dubos, R. J., *Fed. Proc.*, 1946, **5**, 246.

[11] Spratt, N. T., *J. Exp. Zool.*, 1947, **106**, 345.

17232. Effects of 4-Amino-Pteroylglutamic Acid in Dogs with Special Reference to Megaloblastosis.*

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Experimental production of macrocytic anemia and megaloblastosis of bone marrow has been achieved by administration of diets deficient in folic acid (PGA). Miller and Rhoads[1] observed in dogs fed the Goldberger diet macrocytic anemia with megaloblasts in the purple marrow, leucopenia and diarrhea. The same authors described later a hypercellular, megaloblastic marrow in pigs receiving a similar diet.[2] Wills and Stewart[3] reported an experimental macrocytic anemia with megaloblasts in bone marrow in their classical studies with monkeys fed purified diets. In the light of present knowledge the diets employed in the above studies were probably deficient in PGA.[4-9] Subsequent studies in pigs receiving PGA-deficient diets supplemented with a folic acid antagonist, x-methyl-PGA, and sulfasuxidine provided further evidence of the role of folic acid in the maintenance of hematopoiesis. The addition of x-methyl-PGA enhanced the development of macrocytic anemia and leucopenia which responded rapidly to PGA and its conjugates. The bone marrow of deficient pigs showed hyperactive erythropoiesis with megaloblastosis similar to that seen in pernicious anemia of man in relapse.[10,11] With the synthesis of 4-amino-pteroylglutamic acid (4-amino-PGA)[12] an antagonist was provided which proved more potent and more rapid in inducing signs of folic acid deficiency in mice and rats[13-16] than x-methyl-PGA. In view of this finding it was of interest to determine whether the administration of 4-amino-PGA to dogs would also elicit signs of PGA-deficiency similar to the syndrome described by Miller and Rhoads.[1]

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† Post-doctorate fellow of the National Cancer Institute; Neale Research Pathologist, University of Adelaide, S. Australia.

[1] Miller, D. K., and Rhoads, C. P., Proc. Soc. Exp. Biol. and Med., 1933, **30**, 540.

Procedure. Seventeen adult, mongrel dogs were used (Table I). The antagonist‡ was prepared for daily use by dissolving with 2 molar equivalents of $NaHCO_3$ or NaOH to achieve a final pH of less than 8.0. Complete hematological examination including volumetric estimations[17] of blood drawn from the jugular vein into heparinized syringes and of sternal bone marrow aspirations were carried out prior to administration of 4-amino-PGA and at intervals thereafter. The animals were anesthetized briefly with 10 to 20 mg/kg pentothal sodium, intravenously, to facilitate sampling of bone marrow. Blood and marrow films were stained in Jenner-Giemsa buffered at pH 6.85. At autopsy marrow was collected from sternum and femur, smeared, and sectioned. Tissues were taken from all internal organs except nervous system, fixed in Vandergrift's, sectioned, and stained with H and E and Giemsa. In 10 dogs plasma chloride[18] and total nitrogen and NPN in trichloracetic acid filtrates using the micro-Kjeldahl procedure and blood glucose[19] were followed. Plasma protein was calculated to be 6.25 times protein nitrogen.

Clinical course. In dogs 1 through 9 (Table I) receiving single, large doses of 4-amino-PGA no adverse signs were noted until after

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[2] Miller, D. K., and Rhoads, C. P., *J. Clin. Invest.*, 1935, **14**, 153.

[3] Wills, L., and Stewart, A., *Brit. J. Exp. Path.*, 1935, **16**, 444.

[4] Jukes, T. H., and Stokstad, E. L. R., *Physiol. Rev.*, 1948, **28**, 51.

[5] Langston, W. C., Darby, W. J., Shukers, C. F., and Day, P. L., *J. Exp. Med.*, 1938, **68**, 923.

[6] Day, P. L., Langston, W. C., Darby, W. J., Wahlin, J. G., and Mims, V., *J. Exp. Med.*, 1940, **72**, 463.

[7] Day, P. L., Mims, V., and Totter, J. R., *J. Biol. Chem.*, 1945, **161**, 45.

[8] Wilson, H. E., Saslow, S., and Doan, C. A., *J. Lab. Clin. Med.*, 1946, **31**, 631.

[9] Rinehart, J. F., and Greenberg, L. D., *Am. J. Path.*, 1948, **24**, 710.

[10] Cartwright, G. E., Tatting, B., Ashenbrucker, H., and Wintrobe, M. M., *Blood*, 1949, **4**, 301.

[11] Heinle, R. W., Welch, A. D., and Pritchard, J. A., *J. Lab. Clin. Med.*, 1948, **33**, 1647.

[12] Seeger, D. R., Smith, J. M., Jr., Holtquist, M. E., *J. Am. Chem. Soc.*, 1947, **69**, 2567.

[13] Franklin, A. L., Stokstad, E. L. R., and Jukes, T. H., Proc. Soc. Exp. Biol. and Med., 1948, **67**, 398.

[14] Oleson, J. J., Hutchings, B. L., and Subbarow, Y., *J. Biol. Chem.*, 1948, **175**, 359.

[15] Swendseid, M. E., Wittle, E. L., Moersch, G. W., Bird, O. D., and Brown, R. A., *Fed. Am. Soc. Exp. Biol., Proc.*, 1948, **7**, 299.

[16] Philips, F. S., and Thiersch, J. B., *J. Pharm. Exp. Therap.*, 1949, **95**, 303.

‡ The authors are indebted to the Lederle Laboratories Division and the Calco Division of the American Cyanamid Company for liberal supplies of 4-amino-PGA.

[17] Wintrobe, M., "Clinical Hematology," 2nd ed., Lea and Febiger, Philadelphia, Pa., 1946.

[18] Van Slyke, D. D., and Hiller, A., *J. Biol. Chem.*, 1947, **167**, 107.

[19] Nelson, N., *J. Biol. Chem.*, 1944, **153**, 375.

TABLE I.

Effect of 4-Amino-PGA on Cells in Blood and Bone Marrow of Dogs.

	Dosage							Differential count of nucleated cells in bone marrow			Differential count of nucleated erythroid cells in bone marrow				
Dog	mg/kg/day	No. of inj.	Day	Wt, kg	Hcrit., %	PMN	MNC	Myeloid, %	Lymphoid, %	Erythroid, %	Nuclear remnants, %	Normo-blasts, %	Erythro-blasts, %	Megalo-blasts, %	Primitive stem cells, %
1	56iv	1	0	18.0	45.0	6,950	1,650	38	5	57	10	90	0	0	0
			3S	16.3	52.0	700	250	32	5	63	29	50	11	10	0
								19F	2F	79F	50F	23F	10F	17F	0F
2	37iv	1	0	16.2	28.2	14,200	2,500	47	10	43	10	90	0	0	0
			3S	14.8	33.0	2,150	300	29F	21F	50F	51F	24F	0F	25F	0F
4	20iv	1	0	14.1	44.3	6,700	550	89	3	8	6	90	4	0	0
			3S	11.7	60.0	650*		69	3	28	15	34	10	41	0
5	20iv	1	0	12.2	32.9	12,900	1,900	69	8	23	18	80	2	0	0
			3S	11.1	47.0	4,150	200	78	11	11	26	62	4	8	0
								18F	8F	74F	51F	28F	2F	19F	0F
6	20iv	1	0	12.1	32.5	14,600	950	58	3	44	15	80	5	0	0
			3S	10.9	37.0	4,000	450	78	11	11	26	54	4	8	8
								54F	8F	38F	32F	11F	7F	44F	6F
7	10iv	1	0	13.0	46.0	13,100	1,600	65	6	29	3	96	1	0	0
			4S	10.2	58.0	900	300	23	pr	77	29	44	2	25	0
8	10iv	1	0	20.9	41.0	14,600	1,100	66	8	26	2	98	0	0	0
			3	18.3	46.7	5,750	650	68	9	23	28	43	8	21	0
			4S	16.6	60.5	1,850	100	45	2	53	24	40	4	0	32
9	5iv	1	0	16.7	29.2	12,400	2,350	72	4	24	10	90	0	0	0
			3S		43.1	11,700	0	83	7	10	31	43	16	10	0
10	0.1im	5	0	10.5	47.0	19,800	1,250	80	7	13	6	92	2	0	0
			5S	8.5	60.5	9,600	2,100	28	15	57	17	53	0	0	30
12	0.1im	5	0	10.1	36.5	16,600	4,200	40	3	57	5	94	1	0	0
			5S	7.9	58.0	1,600	550	38	7	55	39	36	0	25	0
13	0.05im	14	0	11.3	30.1	10,400	650	46	7	47	13	86	1	0	0
	0.1im	8	14	11.8	39.0	14,200	750	39	6	65	3	97	0	0	0
			21	10.6	43.0	7,350	400	82	2	16	28	72	0	0	0
			24S	9.1	50.0	3,700	100	87F	3F	10F	55F	45F	0F	0F	0F

			0	10.6	51.0	5,550	1,500	48	7	45	19	79	2	0	0
14	0.05im	13	9	10.2	48.0	4,750	600	73	1	26	13	77	5	5	0
			13†	9.2	59.5	11,100	100	81	8	11	2	70	25	0	3
			0	10.6	41.0	7,450	550	54	3	43	10	90	0	0	0
15	0.05im	13	9	8.9	39.0	8,950	1,250	82	7	11	26	67	1	0	6
			13†	7.9	62.0	2,700	200								
16	0.05im	8	0	9.7	44.4	7,850	700	84	6	10	20	76	4	0	0
			7†	8.2	55.0	9,600	400	97	3	pr	8‡	12‡	16‡	0‡	0‡
			0	9.3	30.5	12,200	400	14	5	81	12	88	0	0	0
17	0.05im	6	6S	9.1	46.0	6,850	600	87	5	8	50	25	15	5	5
								92F	2F	8F	56F	21F	0F	9F	14F

* Total leucocyte count per cmm.
† Dogs 14, 16, and 16 succumbed during the 14th, 14th, and 9th day, respectively.
‡ Only 25 cells counted.

F, femoral marrow.
Hcrit, hematocrit.
im, intramuscularly.
iv, intravenously.

MNC, lymphocytes and monocytes per cmm.
PMN, polymorphonuclear granulocytes per cmm.
pr, present.
S, sacrificed.

the first 24 hours when the animals refused food. Diarrhea began shortly after 48 hours. At first yellow fluid, later progressively darker, bloody, and more copious stools, and finally gross blood clots were passed almost continuously. Loss of weight, evident by 48 hours, progressed until time of sacrifice (Table I) when all animals were severely weakened or in coma and dehydrated in appearance. Dog 3 (not shown in Table I) was found dead at 72 hours. In dogs 10 through 17 receiving repeated doses of 4-amino-PGA (Table I) a more prolonged though otherwise similar course was observed. Dog 11 (not shown in Table I) was found dead at 96 hours after receiving 4 daily intramuscular injections of 0.1 mg/kg.

Results. The increased values of the hematocrit noted in Table I confirmed the impression of terminal dehydration. Consistent with this finding was the fact that at time of sacrifice plasma chloride was found decreased 8 to 25 m eq/l below initial values. Simultaneously plasma protein had increased in 8 of 10 dogs. The moribund state of animals 4, 12, and 15 was reflected in elevated plasma non-protein nitrogen, *i.e.*, 92, 107, and 84 mg %, respectively. Changes in blood glucose were not significant.

Neither anemia nor significant changes in erythrocyte volume or content of hemoglobin were observed in animals at any time during the course of intoxication or time of sacrifice.

Reticulocytopenia developed in all but dogs 1 and 8. The peripheral leucocyte count fell in all dogs except 14 and 16 which had granulocytosis due to ascending infections from damaged intestinal tracts. The respective decreases in polymorphonuclear and lymphocytic cells may be seen in detail in Table I.

As noted in aspiration smears and tissue sections of sternal and femoral marrow the total cellularity of the bone marrow decreased involving reduction of both myeloid and erythroid elements. In dogs receiving single, large doses of 4-amino-PGA depletion of bone marrow could be detected after 24 hours and was marked after 48 hours. After 72 to 96 hours only small islands of active hematopoiesis could be found in the fatty, fluid marrow. Differential counts of all nucleated cells and sep-

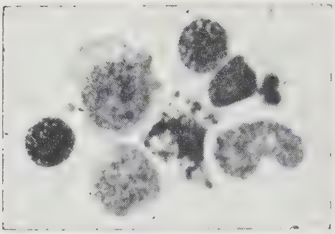

FIG. 1.
Bone marrow aspiration of dog 7 four days after 10 mg/kg of 4-amino-PGA, intravenously. Two megaloblasts, a nuclear "explosion," and a normoblast with nuclear remnant are included in field.

arate differential counts of nucleated erythroid cells ars listed in Table I.

Lesions in myeloid tissues. Peripheral granlocytopenia was associated with depletion of metamyelocytes and neutrophils in bone marrow. However, myelocytes and myeloblasts were uniformly present often maintaining their initial relative proportions in spite of general depletion of myelopoiesis. Only dogs 14 and 16 evidenced active myeloid regeneration. In some cases nests of eosinophils were found in depleted marrows similar to previous findings in rats[16]—an observation still unexplained. Of additional interest was the appearance of giant metamyelocytes, not present prior to administration of 4-amino-PGA, and an increased proportion of hypersegmented neutrophils.

Thrombocytoblasts decreased in numbers with general marrow depletion but were not specifically affected.

Lesions in erythroid tissues. Within 24 to 48 hours after large single doses numerous mature normoblasts were seen to be in pathological mitosis with disintegration of nuclei, chromosomal segregation, and chromatin expulsion into the cytoplasm often simulating nuclear explosions. This process resulted in increased numbers of erythrocytes with nuclear remnants (Fig. 1). During the same period many of the remaining normoblasts and erythroblasts underwent a change in nuclear pattern. The naturally lumpy chromatin was transformed into a distinct network. In the more primitive erythroid elements with basophilic cytoplasm a purple parachromatin appeared consistent with the structure of megaloblasts. Later even hemoglobinized cells with parachromatin were observed. After 48 to 72 hours the proportion of normoblasts was much reduced. In dog 6, for example, at this stage 44% of nucleated erythroid elements in femoral marrow showed nuclear parachromatin and 32%, nuclear remnants. Later with continued depletion primitive stem and sinus endothelial cells become prominent in smears and sections (dogs 6 and 8).

In dogs 10 through 17 megaloblasts were observed in only 3 cases. However, depletion of hematopoiesis was extreme in all dogs receiving repeated injections and megaloblasts might have been present at some stage of intoxication when marrow samples were not taken. The lesions found in dogs 10 through 17 were otherwise identical to those described above.

Lymphoid tissues of spleen, mesenteric nodes, and intestinal tract appeared to be reduced in quantity but no pyknosis or necrosis was seen. By comparison to depleted bone marrows lymphoid tissues were relatively unaffected.

Lesions of the digestive tract were confined to jejunum, ileum, and colon. In all animals at autopsy marked edema and swelling of the mucosa was found throughout small and large intestines. Villi were thickened and blunted by capillary dilatation near the tips. The cytoplasm of superficial epithelial cells was enlarged. In almost the entire colon and ileum epithelium had desquamated leading to denudation of villi and superficial ulceration. In glands and crypts there was evidence of cytoplasmic enlargement and desquamation of epithelium with abnormal regeneration of cells containing irregular nuclei and cytoplasm. Lumina of the deeper glands were frequently filled with plugs consisting of desquamated epithelium and leucocytes. The villi appeared to be matted together resulting from loss of superficial portions and infiltration with leucocytes and fibroblasts below ulcerated areas.

The widespread involvement and necrosis can in large part be attributed to a primary action of 4-amino-PGA on intestinal mucosa. Intestinal damage probably developed early in intoxication and fully explains the bloody diarrhea which was evident 1 to 2 days prior to sacrifice and before hematocrit values were significantly elevated in dogs receiving acutely lethal doses. Ileitis and colitis in the present animals resembled findings in rats in which microscopic evidence of intestinal damage was detected as early as 6 hours after poisoning with fatal doses although death did not ensue for 3 to 4 days.[16] The pathology of the gut was judged to be more extensive and severe than the focal ulceration known to occur in dogs dying of shock.[20-22] Furthermore, unlike the secondary sequellae of local vasoconstriction in shocked animals, focal ulceration did not occur in duodenum, stomach, or esophagus.

With the exception of dogs 14 and 16 no other lesions of significance were detected in other internal organs. The exceptional animals manifested the effects of ascending infections of probable origin in their damaged intestines.

Discussion. The observations described above provide further evidence that 4-amino-PGA acts as an antagonist of PGA and, thereby, confirm previous deductions from studies in rats and mice.[16] The syndrome produced in dogs of ileitis and colitis with diarrhea, peripheral leucopenia, and depletion of bone marrow with appearance of megaloblasts corresponds to observations of the effects of folic acid deficiency in dogs, pigs, and monkeys. The diarrhea and changes in hematopoiesis resemble sprue in man, a disease responding readily to PGA therapy (see recent review[4]).

A comparison of bone marrow from dogs receiving 4-amino-PGA with marrow from cases of pernicious anemia in man reveals certain features in common. These include increased numbers of hypersegmented polymorphonuclears and giant metamyelocytes, megaloblasts and irregularly shaped nuclear remnants in erythrocytes. However, differences between the two marrows are apparent. In pernicious anemia bone marrow exhibits uniform hyperplasia of both erythroid and myeloid elements. In the present dogs general cellularity was reduced and confined to islands of active marrow. In untreated pernicious anemia erythropoiesis is predominantly megaloblastic whereas in the present animals erythropoiesis was a mixture of normoblastic and megaloblastic elements thus corresponding to marrows reported in sprue.[23-28]

The megaloblasts produced in dogs resembled those found in pernicious anemia and sprue as well as those found in humans treated with 4-amino-PGA[29] possibly more closely than the megaloblastic-like cells observed in swine receiving either PGA-deficient diets supplemented with x-methyl folic acid[10] or injections of 4-amino-PGA.[30,31] From studies now available it would appear that megaloblastic response of erythropoiesis to PGA-deficiency varies in different species. Megaloblasts have not been observed in mice and rats,[16,32,33] with the exception of a single report.[34]

Summary. The administration of 4-amino-PGA to dogs produced a sprue-like syndrome with diarrhea, peripheral leucopenia, depletion of bone marrow with increased numbers of hypersegmented polymorphonuclears and giant metamyelocytes, abnormal nuclear dis-

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[20] Blalock, A., *Arch. Surg.*, 1934, **29**, 837.

[21] Klemperer, P., Penner, A., and Bernheim, A. I., *Am. J. Digest. Dis.*, 1940, **7**, 410.

[22] Moon, V. H., Shock. Its dynamics, occurrence and management, Lea and Febiger, Philadelphia, 1942.

[23] Krjukoff, A., *Folia Haematol.*, 1928, **35**, 329.

[24] Ashford, B. K., *Am. J. Trop. Med.*, 1932, **12**, 199.

[25] Makie, F. P., and Fairley, N. H., *Indian J. Med. Res.*, 1929, **16**, 799.

[26] Castle, W. B., and Rhoads, C. P., *Arch. Int. Med.*, 1935, **56**, 627.

[27] Hotz, H. W., and Rohr, K., *Erg. Inn. Med. u. Kindheilk.*, 1938, **54**, 174.

[28] Rodriguez, M., *Puerto Rico J. Pub. Health Trop. Med.*, 1939, **15**, 89.

[29] Thiersch, J. B., *Cancer*, 1949, **2**.

[30] Cartwright, G. E., and Wintrobe, M. M., personal communication.

[31] Thiersch, J. B., and Philips, F. S., unpublished observations.

[32] Endicott, K. M., Daft, F. S., and Ott, M., *Arch. Path.*, 1945, **40**, 364.

[33] Weir, D. R., Heinle, R. W., and Welch, A. D., PROC. SOC. EXP. BIOL. AND MED., 1948, **69**, 211.

[34] Franklin, A. L., Stokstad, E. L. R., Belt, M., and Jukes, T. H., *J. Biol. Chem.*, 1947, **169**, 427.

integration of normoblasts, change of nuclear pattern in erythroid elements and megaloblasts. Doses given were lethal and animals succumbed with anorexia, weight loss, dehydration, and hemorrhagic diarrhea following severe ileitis and ulcerative colitis. It is suggested that the syndrome results from antagonism of folic acid.

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17233. Chronic Toxicity of Gamma Isomer of Hexachlorocyclohexane in the Albino Rat.

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Included in the report by Slade[1] on the discovery of the potent insecticide, "Gammexane," the gamma isomer of benzene hexachloride (γ-BHC)* were investigations of the acute and chronic toxicity of the drug in laboratory rats. His report indicates that the gamma isomer is much more toxic than the α, β, or δ isomers on oral administration, but, when mixed with the diet, levels of γ-BHC up to 30 mg per rat per day had no effect over a 5 week period. Other acute experiments have demonstrated by oral, subcutaneous and intravenous administration, the toxicity of γ-BHC,[2,3] while Laug[4] reported that the gamma isomer was non-toxic when fed in the diet at 20, 500, and 1000 p.p.m. for periods up to 114 days.

Other workers have been interested in the anti-inositol properties of the gamma isomer, since it was reported to resemble the structure of inositol.[1] Their results indicate that the inhibitory effect of γ-BHC is reversed by inositol in the growth of a microorganism,[5] in the α-amylase enzyme system of the pancreas,[6] and in the growing onion root.[7]

A study of transmethylation and lipotropic agents stimulated in this laboratory by the work of Griffith indicated that it might be of interest to examine the anti-inositol and toxic properties of the gamma isomer in the rat, possibly to make available another method of studying inositol metabolism in the intact animal.

Weanling male rats of the St. Louis University strain, 21-24 days old, weighing 44-49 g, were housed in screen cages and allowed food and water *ad libitum*. The animals were weighed twice weekly. The diet to which supplements of γ-BHC and inositol were added was as follows: Casein, 15.0; 1-cystine, 0.3; salts 3A,† 2.0; salts 3B,‡ 2.0; celluration, 2.0; B-complex mixture,§ 1.0; choline chlor-

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[1] Slade, R. E., *Chem. Ind.*, 1945, **64**, 314.

* The term, gamma benzene hexachloride, has been approved by the Committee on Insecticide Nomenclature as the accepted name for the gamma isomer of hexachlorocyclohexane (*Science*, 1949, **109**, 330).

[2] Cameron, G. R., and Burgess, F., Insecticide Development Panel Report (44), 1944, 131.

[3] McNamara, B. P., and Krop, S., *J. Pharm. Exp. Therap.*, 1948, **92**, 140.

[4] Laug, E. P., *J. Pharm. Exp. Therap.*, 1948, **93**, 277.

[5] Kirkwood, S., and Phillips, P. H., *J. Biol. Chem.*, 1946, **163**, 251.

[6] Lane, R. L., and Williams, R. J., *Arch. Biochem.*, 1948, **19**, 329.

[7] Chargaff, E., Stewart, R. N., and Magasanik, B., *Science*, 1948, **108**, 556.

† The salt and vitamin mixtures were devised and thoroughly tested by Dr. Wendell H. Griffith. Since the compositions of these supplements have not been published, the authors wish to express their thanks to Dr. Griffith for his permission to present them here. Salts 3A: $CuSO_4$, 0.5; $CoSO_4 \cdot 7\ H_2O$, 0.1; $ZnSO_4 \cdot 7\ H_2O$, 0.9; $MnSO_4 \cdot 4\ H_2O$, 4.0; $K_2Al_2(SO_4)_3 \cdot 24\ H_2O$, 0.07; NaF, 0.05; Ferric citrate, 24.38; $CaCO_3$, 100; Ca citrate, 185; $MgCO_3$, 45.0; K_2HPO_4, 140 g.

‡ Salts 3B: KI, 0.8; NaCl, 169.2; K_2HPO_4, 100; $CaHPO_4$, 230 g.

TABLE I.
Mortality and Growth Rates of Rats Receiving γ-BHC.

Supplement				
Inositol, mg/g	γ-BHC, mg/g	No. rats	% mortality	Growth, g/wk (avg)
—	—	24	0	28.0
0.5	—	12	0	26.6
2.0	—	8	0	28.9
0	0.8	12	75	11.9
0.5	0.8	24	63	17.1
2.0	0.8	18	72	18.2
0	0.4	16	6	26.0
0.5	0.4	22	36	27.4
2.0	0.4	24	8	28.2

TABLE II.
Mortality and Growth Rates of Littermate Rats Receiving γ-BHC.

	Supplement				Growth rate, g gain/wk		
Group	Inositol, mg/g	γ-BHC, mg/g	No. rats	% mortality	1	2	Avg 4 wks
1	—	—	22	0	24.3	29.6	30.1
2	0.5	—	22	0	25.9	27.6	30.1
3	2.0	—	22	0	24.8	30.0	29.8
4	4.0	—	22	0	26.6	29.8	31.3
5	—	0.4	22	14	10.6	27.6	25.4
6	0.5	0.4	22	14	14.1	29.8	27.9
7	2.0	0.4	22	18	11.6	29.4	27.1
8	4.0	0.4	22	36	15.2	29.7	28.7

ide, 0.4; fat-soluble vitamin mixture,[||] 2.0; lard, 8.0; sucrose, 67.3%.

Supplements of γ-BHC were dissolved in melted lard, while inositol was added at the expense of sucrose. Four week feeding periods were studied throughout. Results of preliminary experiments are presented in Table I.

Control animals on the suboptimal (15%) protein levels gained weight at a rate of 26.6-28.9 g per week. Since the animals receiving no dietary inositol grew as well as those receiving adequate quantities of inositol, it may be stated that with these rations this strain of rat requires no dietary inositol. The presence of γ-BHC in these diets led to markedly diminished growth rates and significant mortality rates, particularly during the first two weeks. Addition of inositol seemed to be without effect. No alopecia, spectacled eye, or other possible signs of inositol deficiency were observed in animals maintained on inositol-free diets for experimental periods up to 8 weeks.

The sequence of events following the administration of sufficient γ-BHC is striking; within 24-48 hours the temperament of the animals alters from docility to ferocity; they react explosively to tactile stimuli, leaping, squealing, and biting as if in pain when touched lightly on the back. They seem to be less sensitive to auditory stimuli, but, superficially, the picture resembles somewhat that seen in magnesium deficiency.[8] Generalized tonic-clonic convulsions are frequent,

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[§] B-Complex mixture: Ca pantothenate, 5.0; niacinamide, 2.5; para-aminobenzoic acid, 1.0; riboflavin, 1.0; pyridoxine • HCl, 0.5; thiamine • HCl, 0.5; biotin, 0.1; "Folvite," 0.05; and sucrose, 989.35 g.

[||] Fat-soluble vitamin mixture: Peanut oil, 195.0; α-tocopherol acetate, 5.0 g. Forty g of this solution is added to cod liver oil, 2.0; corn oil (Mazola), 157.5; 2-methyl-1,4-naphthoquinone, 0.5 g.

[8] Kruse, H. D., Orent, E. R., and McCollum, E. V., *J. Biol. Chem.*, 1932, **96**, 519.

accompanied by hyperpnea and later by hypopnea and exhaustion (some aspects of this syndrome were observed in acute toxicity experiments with γ-BHC[2,3]). Some animals having 2 or more convulsions during the first week have subsequently recovered completely. Of weanling animals failing to survive, death occurred from the second to tenth experimental days.

Additional experiments examined the toxic effects of levels of dietary γ-BHC at 0.6 and 0.2 mg per gram of food. The higher level was lethal to a large proportion of animals, while the lower level was non-toxic and without effect on growth rates. Thus the optimal level of γ-BHC for study was selected at 0.4 mg/g of food; in a similar experiment litter mate controls were used throughout the groups. Growth rates and mortality data are presented in Table II.

The data again demonstrate that this strain of rat does not require supplemental inositol under these experimental conditions. The addition of 0.4 mg of γ-BHC to the diets reduced growth rates markedly during the first week, but during the succeeding 3 weeks, growth rates of surviving animals receiving γ-BHC compared favorably with those of the controls, indicating the development of tolerance. The addition of increasing amounts of inositol seemed to combat slightly the growth depressant effect of γ-BHC (group 5 compared with groups 6, 7 and 8), but had also the effect of increasing mortality. The latter may have been due to a slight increase in food consumption during the first week with a slightly greater intake of γ-BHC in those animals receiving higher levels of inositol. (The first week the controls averaged 7.2 g of food per rat per day, while the food intakes of the animals receiving γ-BHC were: group 5, 4.9; group 6, 5.1; group 7, 5.1; and group 8, 5.3 g of food per rat per day.)

In other experiments the effect of 0.8 mg γ-BHC per gram of food was tested in weanling rats fed a stock laboratory ration, *i.e.*, ground Purina lab chow, to ascertain if the type of diet influenced the reaction to the drug. The young animals exhibited hyperirritability, hyperactivity, convulsions, retarded growth, and a mortality of 25%. Adult rats raised on chow, when placed on the basal ration plus γ-BHC at the 0.8 mg level, lost 10% of their body weight over 10-14 days; in the succeeding two weeks they returned to their starting weights and continued a slow gain. Irritability and infrequent convulsions without mortality resulted.

Animals raised on the highly purified ration (inositol-free) to body weights of 200-300 grams, showed typical symptomatology with a mortality of 50% when the gamma isomer was added to the ration at the level of 0.8 mg per g of food. These animals obtained 3.4-4.1 mg of γ-BHC per rat per day while the weanling rats dying (Table II) received between 1.8-3.1 mg. (Adult rats dying ate 188 mg/kg over a 10-day average survival time, while weanling rats consumed 200 mg/kg body weight over a 4-day average survival period.)

These results are not in accord with those of Slade[1] and Laug[4] whose experimental diets were not reported; Slade reported γ-BHC to be non-toxic when mixed with the diet in amounts up to 30 mg per rat per day, while calculation of the data presented by Laug reveals no toxic effects with dosages up to 20 mg per day. Recently Fitzhugh *et al.*[9] reported that the gamma isomer, when mixed with the diet, is toxic at the level of 0.8 mg per gram of food.

The authors wish to point out that stress was placed upon the rats used in these experiments by suboptimal dietary protein levels in an effort to enhance the action of γ-BHC. Further, animals raised on stock rations tolerated diets containing γ-BHC much better than those raised on experimental diets. These discrepancies may in part be due to the higher nutritive value of normal stock rations, as compared with highly purified rations.

A possible explanation of the failure of inositol to reverse the chronic toxic effects of γ-BHC may be the absence of a demonstrable inositol deficiency in our experiments using rats. A similar failure to demonstrate protection by massive doses of inositol in acute toxicity studies in rabbits has been reported by McNamara and Krop.[10] A recent report by Bijvoet[11] which appeared after our experiments were well under way, indicates lack of similarity in spatial configuration between inositol and γ-BHC. Thus, if this dissimilarity in structure is confirmed, the counteraction of γ-BHC by inositol, previously reported,[5,6,7] may not be the result of specific structural antagonism.

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[9] Fitzhugh, O. G., Nelson, A. A., and Holland, O. L., *Fed. Proc.*, 1949, **8**, 291.

The mechanism of action of γ-BHC is still obscure; after the demonstration by Lane and Williams[6] that inositol is a constituent of α-amylase, the activity of which is inhibited by the gamma isomer, the possibility that hypoglycemia might be involved in the etiology of the convulsions was entertained. Blood sugar levels were determined in a limited series of control and γ-BHC-treated animals and were found within the normal range, even shortly before and after a convulsion.

Gross and microscopic examinations of animals dying after the administration of γ-BHC were performed by Drs. Vincente Moragues and Henry Pinkerton of the Department of Pathology at this institution. No significant pathology was noted. The authors wish to extend their thanks to them.

Conclusion. Gamma benzene hexachloride, mixed with highly purified rations, is toxic to the weanling and adult albino rat. The addition of inositol does not alleviate the toxic symptomatology of γ-BHC.

We wish to thank Mr. Jerome Martin of Commercial Solvents Corporation for his generous gift of γ-BHC, the melting point of which was 112-113°C (uncorr.), slightly higher than the melting points of 4 other samples from various sources.

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[10] McNamara, B. P., and Krop, S., *J. Pharm. Exp. Therap.*, 1948, **92**, 147.

[11] Bijvoet, J. M., *Rec. Trav. Chim.*, 1948, **67**, 777.

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17234. Insulin Stimulation of Glycogen Formation in Rat Abdominal Muscle.*

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Gemmill[1] first demonstrated that insulin stimulated glycogen deposition from glucose in isolated rat diaphragm. Subsequent study with this tissue has disclosed the following important points. Low potassium[2] and high glucose[1] concentrations favor glycogen synthesis. The insulin effect can be demonstrated in diaphragm from either adrenalectomized or hypophysectomized[3] rats and so apparently is independent of the action of hormones from these sources. Desoxycorticosterone, corticosterone and some related substances give reduced glycogen levels when incubated with diaphragm muscle, the effect appearing to be of a glycogenolytic nature and separate from the glycogenic response to insulin.[4]

We have recently examined glucose metabolism in the rat diaphragm with the help of radioactive C^{14} glucose.[5] An increase in the radioactivity of the glycogen in the presence of insulin closely proportionate to the increased glycogen content by analysis provided additional evidence that glucose was the direct precursor of the glycogen rather than a stimulant of glycogenesis from other sources. Insulin pro-

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* This investigation was supported by a research grant from the Division of Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service.

[1] Gemmill, C. L., *Bull. Johns Hopkins Hosp.*, 1940, **66**, 232; 1941, **68**, 329.

[2] Stadie, W. C., and Zapp, J. A., Jr., *J. Biol. Chem.*, 1947, **170**, 55.

[3] Perlmutter, M., and Greep, R. O., *J. Biol. Chem.*, 1948, **174**, 915.

[4] Verzar, F., and Wenner, V., *Biochem. J.*, 1948, **42**, 35, 48.

[5] Bartlett, G. R., Wick, A. N., and MacKay, E. M., *J. Biol. Chem.*, 1949, **178**, 1003.

TABLE I.
Effect of Insulin and Desoxycorticosterone on Glycogen in Isolated Rat Abdominal Muscle.

		Mg of glycogen per 100 mg (wet wt) muscle				
			Incubated 2 hr at 38° under O_2			
Exp.	Days fasted	Initial value	Without glucose	Glucose	Glucose + insulin	Glucose + DOC
1	0	.12	.10	.29	.50	.16
2	0	.16	.19	.35	.54	.30
3	0	.22	.13	.33	.42	.18
4	0	.39	.32	.52	.62	.35
5	0	.22	.21	.37	.59	.36
6	0	.28	.16	.40	.50	.22
7	1	.21	.14	.29	.52	.21
8	1	.15	.16	.32	.38	.17
9	1	.24	.15	.44	.65	.24
10	2	.12	.16	.30	.48	.16
11	2	.21	.14	.32	.45	.20
12	3	.25	.10	.26	.44	.23
Avg		.21	.16	.35	.51	.23

duced a small but significant increase in the radioactive CO_2, hence an acceleration of the combustion of the sugar. Desoxycorticosterone and corticosterone antagonized glycogen formation without appreciably influencing glucose utilization or respiration values and without increasing radioactive carbon dioxide.

The use of insulin in general with isolated tissue slice, mince or extracts, has proved discouraging. Few verifiable effects of any magnitude have been published other than the rat diaphragm work. There has been considerable interest in the reports from Cori's laboratory suggesting that insulin can release some sort of combined pituitary-adrenal cortical inhibition of hexokinase,[6,7] the enzyme responsible for the first step in the utilization of glucose; and unified hypotheses covering the hexokinase action have been offered to explain insulin action *in vivo*.[8] Unfortunately the hexokinase experiments have been ambiguous and difficult to repeat and there appears to be some doubt as to whether even one of the points of action of insulin is a hexokinase inhibitory release.[9,10]

Diaphragm muscle is an important tissue but a specialized muscle with a limited resting period and undergoing continuous rhythmic contractions throughout life. On exploring the possibility of using tissue other than the diaphragm for insulin effects, we have generally found that chopped, shredded or teased muscle loses its ability to synthesize glycogen from glucose. However, we have been able to dissect the internal oblique abdominal muscle of the rat with a minimum of tearing and find that this muscle *in vitro* will give considerable glycogen synthesis from added glucose and also a further large increase of glycogen in the presence of very small amounts of insulin. As in the case of the diaphragm less glycogen is found in the presence of desoxycorticosterone. Results of experiments with this tissue are recorded in Table I. 400 - 500 mg wet weight of abdominal muscle was incubated in 3.0 ml of pH 7.4 Ringer phosphate with or without 0.2 molar glucose, 0.1 unit per ml of insulin and 10 μg per ml of desoxycorticosterone.† Flasks were shaken for 2 hours in an oxygen atmosphere. Glycogen was assayed by the usual alkali digestion-alcohol precipitation

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[6] Colowick, S. P., Cori, G. T., and Slein, M. W., *J. Biol. Chem.*, 1947, **168**, 583.

[7] Price, W. H., Cori, C. F., and Colowick, S. P., *J. Biol. Chem.*, 1945, **160**, 633.

[8] Stetten, D., Jr., *J.A.M.A.*, 1946, **132**, 373.

[9] Broh-Kahn, R., and Mirsky, I. A., *Science*, 1947, **106**, 148.

[10] Stadie, W. C., and Haugaard, N., *J. Biol. Chem.*, 1949, **177**, 311.

† Pure Zn insulin was obtained through the courtesy of Dr. Edwin E. Hays of Armour and Co., and desoxycorticosterone from Dr. Edward Henderson of the Schering Corp.

method.[11] 100 - 120 g female albino rats were used and that portion of each internal oblique muscle which could be separated as a thin layer without appreciable trauma was placed intact in the incubating flask. When the muscle was cut into a series of thin strips, little or no glycogen synthesis took place.

The results reported here are evidence that insulin-glycogenesis stimulation *in vitro* is not peculiar to diaphragm muscle. New material is available for further investigation of the action of insulin and other factors controlling glycogen formation and utilization.

Summary. If used in thin sheets with a minimum of tissue injury isolated skeletal muscle in the form of the internal oblique muscle of the rat forms more glycogen under the influence of insulin just as does the specialized muscle comprising the diaphragm. Desoxycorticosterone reduces the amount of glycogen formed by incubation with glucose alone as in the case of diaphragm muscle. Skeletal muscle offers new material for further investigation of the action of insulin.

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[11] Good, C. A., Kramer, H., and Somogyi, M., *J. Biol. Chem.*, 1933, **100**, 485.

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17235. Normal and Seizure Levels of Lactate, Pyruvate and Acid-Soluble Phosphates in the Cerebellum and Cerebrum.*

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Seizures induced by various means have been shown to result in an increase in lactic acid, pyruvic acid and inorganic phosphorus and a decrease in high energy phosphate levels in the cerebral cortex[1,2] or whole brain[3] of various experimental animals. There is little data on the several parts of the brain. As reported previously it is possible to show that seizures induced by low doses of nitrogen mustards start first in the cerebellum; further, the convulsive activity of these compounds is potentiated by inhalation of carbon dioxide.[4] Inhalation of carbon dioxide tends to prevent the changes in lactic acid and phosphate attendant upon seizures.[5] As a sequel to the above studies, seizures were induced in a series of cats using representative nitrogen mustards and various other convulsants, the brains were frozen *in situ* and the cerebrum and cerebellum then analyzed separately for lactic acid, pyruvic acid, inorganic phosphorus, and high energy phosphates.

Experimental. Preparation of the animals, methods of EEG and EKG recording, procedure of freezing with liquid air and methods of analysis have been reported in detail in previous papers from this laboratory.[1,5] Seizures were induced by intravenous injections of the compounds indicated in Table I. Cerebral and cerebellar cortex were easily dissected from the frozen brains with a small chisel and mallet. During the dissection frequent applications of liquid air were used to maintain the tissue in a frozen state.

Results. The data are summarized in Table I. In normal brains frozen without seizures the levels of the constituents determined were very similar in both cerebellum and cerebrum and compared well with those previously reported[1,5] except that the phosphocreatine levels were somewhat low. In brains frozen during seizures, however, marked dif-

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* Aided in part by grants from the Rockefeller Foundation and the Miller Epilepsy Fund.

[1] Klein, J. R., and Olsen, N. S., *J. Biol. Chem.*, 1947, **167**, 747.

[2] Stone, W. E., Webster, J. E., and Gurdjian, E. S., *J. Neurophysiol.*, 1945, **8**, 233.

[3] LePage, G. A., *Am. J. Physiol.*, 1946, **146**, 267.

[4] Pollock, G. H., and Bain, J. A., in preparation.

[5] Bain, J. A., and Klein, J. R., *Am. J. Physiol.*, Sept., 1949. **158**.

TABLE I.
Normal and Seizure Levels of Lactate, Pyruvate and Acid-Soluble Phosphates in the Cerebellum and Cerebrum.

Lactate*		Pyruvate		Inorg. P		Phosphocreatine		Adenosine Tri-PO_4		Adenosine Di-PO_4		Remarks
Cb†	Cx	Cb	Cx	Cb	Cx	Cb	Cx	Cb	Cx	Cb	Cx	
Controls.												
1.37	1.36	.069	.077	6.72	5.11	1.58	1.49	0.81	0.48	2.56	2.70	
1.50	1.40	.120	.136	4.70	4.93	2.10	1.95	1.30	0.90	1.85	2.06	
1.43	1.38	.095	.106	5.71	5.02	1.84	1.72	1.06	0.69	2.20	2.38	Avg
Nitrogen Mustards.‡												
1.80	4.30	.177	.213	4.17	4.24	2.10	1.20	0.39	1.69	3.15	1.32	$C_3H_7N(C_2H_4Cl)_2$
3.48	5.86	.193	.268	3.84	3.60	2.28	1.91	1.43	0.85	1.82	2.51	$H_2C=CHN(C_2H_4Cl)_2$
1.66	5.64	.134	.208	4.56	4.20	2.29	1.92	1.13	2.25	2.19	1.00	$C_6H_5CH_2N(C_2H_4Cl)_2$
1.18	2.76	—	—	4.08	4.56	2.77	1.69	1.71	1.49	1.73	1.57	$OC_4H_8NC_2H_4Cl$
3.45	4.08	.277	—	5.19	3.58	1.38	2.30	1.58	1.46	1.97	1.60	$C_4H_9N(C_2H_4Cl)_2$
1.86	2.10	.132	.146	5.94	4.56	3.08	2.16	1.59	0.79	2.00	2.10	$C_3H_7N(C_2H_4Cl)_2$
3.82	7.60	.250	.280	3.72	3.48	3.13	2.03	—	1.36	3.15	1.77	$(C_2H_4Cl)_2NCH_2CH_2N(C_2H_4Cl)_2$
2.46	4.62	.194	.227	4.50	4.03	2.43	1.89	1.30	1.41	2.30	1.70	Avg
Metrazol.												
1.37	2.42	.087	.100	5.18	4.83	2.52	1.50	0.38	0.52	3.48	2.73	
1.50	2.66	.104	.110	4.97	4.68	1.87	1.20	0.53	0.71	2.84	2.35	
1.43	2.54	.095	.105	5.07	4.53	2.19	1.35	0.45	0.62	3.16	2.54	Avg
Aminophylline.												
2.76	9.72	.116	.145	5.88	6.54	1.52	0.42	0.42	0.58	3.00	2.96	16′ convulsion
1.56	6.06	.154	.293		(8.21	5.88)		0.75	1.10	3.11	2.18	13′ ”
4.34	4.08	.126	.156	5.90	5.64	0.82	0.42	0.11	0.40	3.02	2.70	8′ ”
3.96	9.72	.112	.106	5.94	6.74	0.60	0.08	0.33	0.56	2.90	2.74	4′ ”
3.16	7.40	.127	.175	6.48	6.20	0.99	0.31	0.40	0.66	3.01	2.64	Avg
Various.												
2.64	3.51	.240	.130	4.92	3.96	2.05	1.81	1.42	2.28	2.19	0.97	Castorix‖
1.62	3.48	.126	.164		(6.48	5.62)§		1.62	1.52	1.85	1.78	Dibenamine
2.68	4.50	.188	.254		(6.72	5.36)		1.16	1.64	2.24	1.42	Br. Camphor
5.74	6.04	.259	.339		(6.12	5.02)		—	—	—	—	Coramine
2.74	3.60	.164	.206		(7.10	5.18)		1.44	1.16	2.18	1.74	Strychnine
2.20	4.08	.115	.156	3.90	4.14	2.46	2.22	0.92	0.80	1.86	2.30	Electric shock

* All values mM/1000 g (wet wt).

† "Cb" and "Cx" designate cerebellar and cerebral cortex, respectively.

‡ Obtained through the courtesy of the University of Chicago Toxicity Laboratory.

§ Pairs of figures enclosed in parentheses are the sum of inorganic phosphate and phosphocreatine for cerebellum and cerebrum, respectively.

‖ 2-Chloro 4-dimethyl 1,6-methyl pyrimidine.

ferences were found. The lactic acid level rose only about half as much and the phosphocreatine level was appreciably higher in the cerebellum as compared to the cerebrum. There were no systematic differences discernible in the inorganic phosphorus and ATP-ADP levels; however, this may be due to the fact that many of the seizures were potentiated with carbon dioxide[4] a procedure which tends to minimize changes in the assayed constituents during seizures.[5] These results obtained despite the fact that seizure activity was as violent in the cerebellum as in the cerebrum as judged from the EEG recordings.

Discussion. The results reported above point to a quantitative if not qualitative difference in the metabolism of the cerebellum and the cerebrum under the stress of seizures. It is not surprising that this should be so since there are marked histological differences between the two parts. Furthermore, the various parts of the central nervous system have been shown to differ in their sensitivity to cyanide,[6] their carbonic anhydrase activity,[7] their cholinesterase activity,[8] and many other particulars.

Summary. Lactic acid, pyruvic acid, inorganic phosphorus, phosphocreatine, and ATP-ADP were determined in liquid air fixed brains of normal and convulsed cats. In normal brains the levels in cerebellum and cerebrum were approximately the same. In convulsed brains, however, the lactic acid increases were much smaller and phosphocreatine levels were higher in cerebellum than in cerebrum despite the fact that seizure activity was approximately of equal intensity in both parts.

The authors wish to thank Miss Ruth Hurwitz for valuable technical assistance.

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[6] Ward, A. A., and Wheatley, M. D., *J. Neuropath. and Exp. Neurol.*, 1947, **6**, 292.

[7] Ashby, W., *J. Biol. Chem.*, 1944, **152**, 235.

[8] Augustinsson, K. B., *Acta Physiologica Scandinavica*, 1948, **15**, suppl. 52, 8.

17236. Lactate, Pyruvate, and Acid-Soluble Phosphates in Monkey Brains Treated with Carbon Dioxide and Electric Shock.*

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(Introduced by Warren S. McCulloch.)

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There now exists in the literature a considerable body of data regarding the effect of seizures upon the lactate, pyruvate, and acid-soluble phosphate levels in the brains of dogs,[1] rats,[2] and cats[3,4,5] under various conditions. It seemed, therefore, desirable to extend these analyses to animals higher in the phylogenetic scale, including man. As a step toward this end, a limited series of monkeys has been studied in the normal state, treated with carbon dioxide,[4] electric shock, and electric shock plus carbon dioxide.[6]

Methods and Results. Treatment of the animals, brain wave recordings, and analysis of the liquid air fixed brains were carried out as described in previous reports from this laboratory.[3-6] The results are summarized in Table I. Comparison with the data on other animals shows that the levels of all constituents assayed are of the same magnitude in monkeys as in dogs, cats, and rats and the changes brought about by seizures and carbon dioxide inhalation are in the expected direction.

Discussion. The results reported show that it is probably justifiable to extend conclusions drawn from data on experimental animals to man, as far as the brain constituents here assayed are concerned, since it seems unlikely that there would be a sharp change between man and monkey when monkey does not differ from other mammals.

Another point which is clearly demonstrated by a comparison of animals 3 and 4 is that the rise in lactate, pyruvate, and inorganic phosphate elicited by seizures is the result of the hyperactivity of the brain and not of the stimulus which initiates said hyperactivity. There is an indication in the data that the directly driven response to the stimulus alone, without seizures, may cause a fall in phosphocreatine (animal 4) and possible adenosine triphosphate; but in the case of the latter

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* Aided in part by grants from the Miller Epilepsy Fund and the Rockefeller Foundation.

[1] Stone, W. E., Webster, J. E., and Gurdjian, E. S., *J. Neurophysiol.*, 1945, **8**, 233.

[2] LePage, G. A., *Am. J. Physiol.*, 1946, **146**, 267.

[3] Klein, J. R., and Olsen, N. A., *J. Biol. Chem.*, 1947, **167**, 747.

[4] Bain, J. A., and Klein, J. R., *Am. J. Physiol.*, Sept., 1949, **158**.

[5] Bain, J. A., and Pollock, G. H., in preparation.

[6] Stein, S. N., and Pollock, G. H., PROC. SOC. EXP. BIOL. AND MED., 1949, **70**, 290.

TABLE I.
Lactate, Pyruvate, and Acid-Soluble Phosphates in Monkey Brain.

Animal No.	Lactate		Pyruvate		Inorg. P		Phosphocreatine		Adenosine Tri-PO_4		Adenosine Di-PO_4		Treatment
	Cb*	Cx	Cb	Cx	Cb	Cx	Cb	Cx	Cb	Cx	Cb	Cx	
1	1.68†	1.80	0.171	0.157	3.65	3.93	1.70	1.33	1.40	1.68	1.74	1.27	Control
2	1.44	1.41	0.078	0.083	4.20	4.71	1.27	1.15	1.51	1.43	1.76	1.91	30% CO_2, 70% O_2 for 3 min.
3	5.22	5.40	0.263	0.269	6.32	5.67	0.47	1.12	1.14	1.18	1.73	1.65	10 sec. electric shock 55 sec. seizure
4	1.74	1.95	0.163	0.168	4.44	4.62	0.62	0.54	1.09	1.34	1.33	1.11	30% CO_2, 70% O_2 for 3 min. 10 sec. electric shock 55 sec. lag period No seizure

* "Cb" and "Cx" designate cerebellar and cerebral cortex, respectively.
† All values expressed as mM/1000 g (wet wt) tissue.

compound, the changes are too small, considering the precision of the methods of analysis, to allow conclusions to be drawn without more extensive studies on a larger series of animals. Such a study on monkeys is, at present, beyond our means.

Summary. The brains of normal monkeys and those treated with carbon dioxide and electric shock were fixed with liquid air and analysed for lactate, pyruvate, and acid-soluble phosphates. The levels of these constituents and their change under the above conditions were shown to be of the same magnitude and in the same direction as those found in similarly treated dogs, cats, and rats.

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17237. Effect of Environmental Temperature on the Response of Mice to Whole-Body Roentgen Radiation.

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A low environmental temperature for frogs and new-born rats is accompanied by decreased body temperature and oxidative metabolism, and relatively low radiosensitivity.[1-3] In the mouse, when the temperature regulating mechanism is functioning normally, a cold environment produces only a slight depression of body temperature, and oxygen consumption is elevated.[4,5] This increase in oxidative metabolism has been attributed to increased thyroid activity.[6,7] Since thyroid

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[1] Patt, H. M., Swift, M. N., and Tyree, E. B., *Fed. Proc.*, 1948, **7**, 90.

[2] Patt, H. M., Swift, M. N., and Tyree, E. B., *Quart. Rep.*, May-Aug., 1947, A. M. Brues, Editor, AECD 2024, 57.

[3] Evans, T. C., Goodrich, J. P., and Slaughter, J. C., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 434.

[4] Turner, M. L., *Am. J. Physiol.*, 1948, **152**, 197.

[5] Harrington, L. P., *Am. J. Physiol.*, 1940, **129**, 123.

[6] Hurst, V., and Turner, C. W., *Am. J. Physiol.*, 1947, **150**, 686.

[7] Dempsey, E. W., and Astwood, E. B., *Endocrinology*, 1943, **32**, 509.

feeding has been shown to increase the incidence of death following roentgen irradiation in mice,[8] experiments were carried out to determine whether or not a cold environment would produce the same effect.

Male mice of N.I.H. stock were received at weaning, divided into treatment groups in which each litter was represented as equally as possible, and placed on a diet of Purina pellets. Three weeks later they were irradiated in groups of 10 made up of approximately equal numbers from each treatment group. The experiments were terminated 4 weeks after irradiation, 98.7% of the observed deaths having occurred between 4 days and 3 weeks.

From one experiment to another we have been unable to duplicate mortality for a given radiation dose, due possibly to genetic heterogeneity of the stock, possibly to fluctuations in X-ray output. Within an experiment these variations should have been largely neutralized by the equal distribution of litters and treatments into radiation groups. Only relative mortality figures are comparable between experiments, and only balanced experiments can be summed.

Irradiation factors were: 170 Kv, 20 ma, added filtration 0.55 mm of aluminum and 0.25 mm of copper, focal distance 50 cm, and dose rate 56 r to 64 r per minute. Total doses were 500 r in the first experiments, 470 r in the last 2 experiments. Cold and hot rooms, thermostatically controlled, were maintained between the limits 9 to 11°C and 29 to 30°C. The 30° temperature was chosen because, according to Harrington,[5] heat production is minimal at an environmental temperature of 30° to 33°C.

Paraffin sections of formalin-fixed tissues were stained with azure eosinate.[9] Frozen sections were stained for fat with oil red 0 by the method of Lillie and Ashburn.[10] With this method little or no fat is extracted from the tissue by the staining solution.

In the first 6 experiments a total of 200 mice kept at 30° and 256 at 10°, irradiated with 500 r, were caged together in groups of 10 on sawdust. At each temperature approximately half of the mice were acclimatized for 2 weeks prior to irradiation and half were placed at the temperature immediately after irradiation, where they remained throughout the observation period. In the 30° environment 46% of those acclimatized died compared with 63% of those not acclimatized. At 10°, 52% of those acclimatized died compared with 71% of those not acclimatized. The χ^2 values of the totals do not indicate significant differences between the temperature groups (2.7 for acclimatized and 1.8 for non-acclimatized mice). Acclimatization at either temperature favored survival $\chi^2 = 5.4$ for 30°, 8.3 for 10°). The results of the individual experiments were very erratic, however, and thus afford little confidence in the χ^2 values of the totals.

In this group of experiments survivors at either temperature gained weight during the last 2 weeks of observation. Leucocyte counts made on the first and fourth days after irradiation showed no difference attributable to temperature. Of the non-irradiated controls kept at these temperatures 2 out of 79 at 10° and 1 out of 50 at 30° died from undetermined causes, while the others made satisfactory gains in weight.

Histopathologic changes in the bone marrow, spleen, lymph nodes, thymus, and testis were essentially similar to those recorded in the literature,[11,12] often varied markedly among mice receiving the same treatment, and the severity of the changes did not consistently favor any one treatment group.

In the last 3 experiments the mice were caged singly in suspended mesh cages without sawdust from the time of irradiation (Table I). Ten non-irradiated controls so caged in the 10° environment survived the observation period of 4 weeks without apparent ill ef-

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[8] Blount, H. C., Jr., and Smith, W. W., *Science*, 1949, **109**, 83.

[9] Lillie, R. D., Histopathologic Technic, 1949, Blakiston Co., Philadelphia, Pa.

[10] Lillie, R. D., and Ashburn, L. L., *Arch. Path.*, 1943, **36**, 432.

[11] Brecher, G., Endicott, K. M., Gump, H., and Brawner, H. P., *Blood*, 1948, **3**, 1259.

[12] Henshaw, P. S., *J. Nat. Cancer Inst.*, 1944, **4**, 485.

TABLE I.
Incidence of Death Following Irradiation of Mice Caged Singly at 10° and 30°C.

Exp. No.	Dose r	30°C				10°C				χ^2 corrected			
		Acclimatized		Not accl.		Acclimatized		Not accl.		Acclimatization		Temperature	
		Dead	Alive	Dead	Alive	Dead	Alive	Dead	Alive	at 30°	at 10°	Accl.	Not accl.
7	500	8 40%	12	16 80%	4	16	4	20 100%	0	5.10	2.50*	5.10	2.50*
8	470	—	—	16 27%	44	—	—	41 68%	19	—	—	—	19.24
9	470	—	—	23 38%	37	—	—	36 60%	24	—	—	—	4.80
Totals				55 39.3%	85			97 69.3%	43				24.18

* These values are inexact because 100% of the non-acclimatized mice at 10° died.

fects. Rectal temperatures were determined by an oral clinical thermometer with the bulb completely inserted into the rectum while the mouse was restrained by a wire jacket. Mice at the end of 4 weeks in the 30° environment had an average temperature of 38.4°C, 1.2° higher than those in the 10° environment.

In these experiments, where the mice were unable to huddle together, 69% in the 10° room died compared with 39% in the 30° room. The χ^2 values for individual experiments are relatively homogeneous, and the value 24.18 for the totals indicates a high degree of probability that the results were not due to chance alone. In the one experiment where acclimatization effect was studied acclimatization again appeared to favor survival.

The detrimental effect of the cold environment was clearly demonstrable when the mice were not allowed to huddle together. The conclusion that resistance to lethal X-ray effects is lowered in a cold environment, when the oxidative metabolism of mice is elevated, is in harmony with results from feeding desiccated thyroid. There are, however, other physiological variables, such as the distribution of blood between peripheral and visceral circulation, which may or may not alter the resistance of the organism to irradiation.

In gross examination of the mice that died following irradiation a uniformly pale or mottled liver was frequently observed in those from the 30° environment. Where 10 mice were caged together, 48 out of 64 at 30° showed this condition in contrast to 7 out of 76 mice at 10°. Among the mice caged singly, 31 out of 41 had pale or mottled livers at death in contrast to 6 out of 107 mice of the 10° groups. There was no apparent difference in this respect between acclimatized and non-acclimatized mice. The liver was examined microscopically in mice caged singly that died 7 to 14 days after irradiation. From 27 such mice at 30°, 19 showed marked and 4 showed moderate fatty changes, while from 30 such mice at 10° no livers showed marked and 4 showed moderate changes. Among control mice caged 10 together only one out of the 10 kept 2 weeks at 30° showed moderate fatty changes, and none at 10° showed such changes.

The higher incidence of pale, fatty livers in mice that died in the 30° environment was not obviously dependent upon the occurrence of hemorrhage as grossly observed, or upon differences in hemoglobin concentration as determined on 22 mice 11 days after irradiation (at 30°, mean hemoglobin 10.3 g per 100 ml; at 10°, 10.5 g per 100 ml). Since the change occurred chiefly in the groups having the lower incidence of death it is apparently not associated with low resistance to irradiation. The many non-specific factors that could conceivably be responsible for the fatty changes do not permit the conclusion that irradiation had more than an indirect effect in its induction.

Summary. The net effect of a sufficiently cold environment is to lower the resistance of mice to the lethal action of X-rays.

Subjection to a 10° or 30°C environment for 2 weeks prior to irradiation favors survival of irradiated mice maintained in those environments.

Mice kept in a 30° environment that die following irradiation frequently show fatty changes in the liver.

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17238. Effect of Nitrocompounds on Viruses of the Psittacosis-Lymphogranuloma Group.

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Because of the observation that nitroacridines showed a marked inhibition of agents of the psittacosis-lymphogranuloma group[1,2] while chloro-substituted and certain other acridines were ineffective,[1] it seemed desirable to investigate the activity of nitrocompounds other than acridines. Substances containing a benzene or furan ring were selected for investigation because certain structural features in nitroacridine are also present in the latter, simpler compounds. While these studies were in progress other publications have described activity of two compounds containing a nitro-phenyl group against viruses of the psittacosis group or rickettsiae. One of these is chloramphenicol (chloromycetin)[3,4] and the other a nitro analogue of D.D.T., 1,1, 1-trichloro-2, 2-bis (para-nitrophenyl) ethane which was found to show some effectiveness against murine typhus in mice.[5]

Material and methods. The source of the strains of the viruses of lymphogranuloma venereum, meningopneumonitis, cat pneumonitis, and mouse pneumonitis is given in a previous publication.[1] Experiments in mice 16 to 18 grams in weight were done with mouse lung passage of these viruses inoculated by the intranasal route. Titrations were done and the infecting dose adjusted so that control mice in each experiment when killed on the 6th day had pulmonary lesions giving a score between 30 and 60.† Drugs were given by the intraperitoneal route as single daily doses starting 2 hours after the virus. Control mice received saline intraperitoneally.

The experiments in chick embryos were done with yolk sac passages of the four viruses inoculated either into the yolk sac or the allantoic sac. The technic of the experiments using inoculation into the yolk sac with about 10 LD_{50} was identical with that previously described.[1] In the other series of experiments 10 to 100 ID_{50} (50% infectious doses) were inoculated into the allantoic sac of 11-day-old chick embryos and a single dose of drug inoculated after the virus either into the allantoic sac or into the yolk sac. Control embryos received saline. The embryos were sacrificed on the 5th day after inoculation and the degree of viral multiplication in the allantoic sac determined by inoculating allantoic fluids from individual treated and control eggs into mice by the intranasal route. Fluids

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* Fellow of the American Bureau for Medical Aid to China, Inc.

[1] Eaton, M. D., vanAllen, A., and Weiner, A., Proc. Soc. Exp. Biol. and Med., 1947, **66**, 141.

[2] Hurst, E. W., *Brit. J. Pharm. and Chemotherap.*, 1948, **3**, 181.

[3] Smadel, J. E., and Jackson, E. B., *Science*, 1947, **106**, 418.

[4] Bartz, Q., Paper presented at Second National Symposium on Recent Advances in Antibiotic Research; also *J. Am. Chem. Soc.*, 1949, in press.

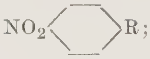
[5] Fitzpatrick, F. K., Proc. Soc. Exp. Biol. and Med., 1949, **70**, 90.

† A lesion score of 100 represents death with complete pulmonary consolidation. In surviving mice 80 represents on the average 4+ consolidation; 60, 3+; 40, 2+; and 20, 1+.

TABLE I.
Effect of Substituted Nitrobenzenes on Viral Respiratory Infections in Mice.

Virus	Substituent R*	Daily dose, mg	No. of mice	Lesion score T/C†	Lesion score % change‡	Lung wt (g) T/C†	Lung wt (g) % change‡
Cat pneumonitis	COONa	10	43	29/41	—29	0.248/0.288	—14
	$CONH_2$	5	18	24/40	—40	0.192/0.247	—23
	$C(NH)NH_2$	2	40	30/40	—25	0.284/0.313	— 9
	SO_2NH_2	4	23	25/52	—52	0.221/0.305	—27
	(Sulfanilamide)	10	14	42/46	—11	0.290/0.293	— 1
	Chloramphenicol	2	16	36/38	— 5	0.402/0.340	+18
	,,	10	16	47/73	—36	0.374/0.460	—19
Mouse pneumonitis	COONa	10	25	39/34	+14	0.295/0.269	+10
	$CONH_2$	5	20	35/31	+13	0.220/0.255	—14
	$C(NH)NH_2$	2	53	33/38	—13	0.312/0.305	+ 2
	SO_2NH_2	4	21	18/52	—65	0.168/0.260	—35
	(Sulfanilamide)	10	13	20/57	—65	0.173/0.273	—37
	Chloramphenicol	2	16	41/46	—11	0.282/0.376	—24
Lymphogranuloma venereum	COONa	7.5	27	38/39	— 3	0.261/0.266	— 2
	$CONH_2$	5	16	36/40	—10	0.244/0.280	—14
	Chloramphenicol	2	16	23/41	—41	0.204/0.345	—41
Meningopneumonitis	COONa	7.5	26	33/35	— 6	0.255/0.271	— 6
	$CONH_2$	5	33	26/32	—19	0.312/0.323	— 3
	SO_2NH_2	3	31	39/46	—16	0.348/0.379	— 8
	Chloramphenicol	2	16	37/51	—28	0.387/0.423	— 9
	,,	5	16	21/42	—50	0.295/0.456	—35

* Substituents in para position to nitro group:



R; for chloramphenicol R = $CH(OH)$
$CH(NH_2COCHCl_2)$
CH_2OH

† T = Treated mice; C = Control mice.
‡ % change = 100 (—1 + T/C).

were considered to be significantly infected when they produced average lesion scores of 20 or over in the group of 3 mice subinoculated from each embryo.

Drugs were injected by the intraperitoneal route in mice without heating or filtration, insoluble drugs being ground with a small amount of starch and suspended in saline. For inoculation of chick embryos the drugs were sterilized in saline suspension or solution by heating in a boiling water bath for 20 minutes. One nitrofuran which was soluble but unstable at 100°C was sterilized by filtration through a fritted glass filter. Dosage up to one-half or one-third of the maximum tolerated dose was used in these experiments.

Results in mice. These were expressed as reduction of the observed gross pulmonary consolidation and the weight of the lungs of the treated mice as compared with the controls. Further details on the measurement of the lung weights and their relation to the degree of consolidation will be found in another publication.[6] In most instances two or three experiments were done with the same drug and when the effects in each experiment were similar the results were averaged. A summary of the findings in mice with compounds containing a nitrophenyl group is presented in Table I. With four compounds, sodium p-nitrobenzoate, p-nitrobenzamide, p-nitrobenzamidine, and p-nitrobenzene sulfonamide, evidence of inhibition of the cat pneumonitis virus was obtained. The most significant effects were those with the two acid amides. With the exception of p-nitrobenzene sulfonamide these substances had no effect against the agent of mouse pneumonitis. Since the latter virus is sensitive to the action of sulfanilamide the positive result with p-

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[6] Huang, C., and Eaton, M. D., *J. Bact.*, 1949, in press.

TABLE II.
Effect of Nitrofurans on Viral Respiratory Infections in Mice.

Virus		Substituent R*	Daily dose, mg	No. of mice	Lesion score		Lung wt (g)	
					T/C	% change	T/C	% change
Cat pneumonitis	(I)	$CH{=}N{-}\overset{H}{N}{-}\overset{O}{C}{-}\overset{O}{C}{-}NH_2$	3	22	31/48	—35	0.228/0.353	—35
	(II)	$CH{=}N{-}\underset{CH_2{-}CH_2OH}{\overset{H}{N}}{-}CONH_2$	2	15	20/45	—56	0.236/0.393	—40
Mouse pneumonitis	(I)	$CH{=}N{-}\overset{H}{N}{-}\overset{O}{C}{-}\overset{O}{C}{-}NH_2$	3	27	26/56	—54	0.201/0.312	—36
Lymphogranuloma venereum	(I)	$C{=}N{-}\overset{H}{N}{-}\overset{O}{C}{-}\overset{O}{C}{-}NH_2$	3	27	6/51	—88	0.178/0.290	—39
	(IV)	$CH(CO_2CH_3)_2$	2	26	18/37	—51	0.217/0.301	—28
Meningo-pneumonitis	(I)	$C{=}N{-}\overset{H}{N}{-}\overset{O}{C}{-}\overset{O}{C}{-}NH_2$	3	36	11/36	—70	0.211/0.301	—30
			2	16	18/51	—41	0.333/0.423	—21
	(III)	$C{=}N{-}\overset{H}{N}{-}\overset{O}{C}{-}NH_2$	3	31	21/36	—42	0.238/0.340	—30
	(II)	$C{=}N{-}\underset{CH_2CH_2OH}{N}CONH_2$	2	20	26/40	—35	0.288/0.379	—24
	(IV)	$CH(CO_2CH_3)_2$	2	20	41/49	—16	0.372/0.418	—11

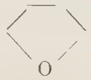
* All are derivatives of 5-nitro-2-furaldehyde NO_2  —R where R represents the aldehyde group combined as semioxamazone (I) 2-β-hydroxyethyl-semicarbazone (II), semicarbazone (III), or diacetate (IV).

TABLE III.
Effect of Nitro Compounds on Allantoic Infections with Cat Pneumonitis Virus in Chick Embryos.

Drugs			Degree of infection as indicated by subinoculation of allantoic fluid into mice[†]			
Name	Dose, mg	Route	LS<20	LS20-50	LS>50	Average L.S.
Sodium p-nitrobenzoate	10	A	11	6	2	27
p-Nitrobenzamide	10	YS	4	3	1	25
Saline		A	3	9	16	78
p-Nitrobenzene sulfonamide	1	A	6	8	1	22
(Sulfanilamide)	1	A	2	10	3	37
Saline		A	0	10	5	47
NF II[‡]	0.5	YS	6	3	1	24
Chloramphenicol	1	YS	6	2	0	7
Saline		A	4	6	2	31
(Penicillin G)	0.3	YS	14	3	3	18
Saline		YS	3	3	9	65

[*] A = Allantoic; YS = Yolk sac.

[†] Eggs are divided into three groups; number not significantly infected, lesion score (LS) <20 in mice; number moderately infected, LS20-50; number with heavy infection, LS>50. Average lesion score for entire group of eggs is given in last column.

[‡] NF II = 5-nitro-2-furaldehyde-2-β-hydroxyethyl semicarbazone.

nitrobenzene sulfonamide may be attributed to reduction of the nitro group to an amino group in the body. The data from a comparable experiment with sulfanilamide included in Table I show that the effect was very similar in degree. It will be seen, however, that sulfanilamide had little effect on the cat pneumonitis virus while the various nitro-compounds showed slight or moderate activity. With p-nitrobenzamide and p-nitrobenzene sulfonamide, the reduction of pulmonary lesions in mice infected with the agents of lymphogranuloma venereum and meningopneumonitis were very slight and in most cases probably not significant.

Chloramphenicol[‡] at a dosage of 2.0 mg/mouse daily had no significant effect against the virus of cat pneumonitis, and only a slight effect against the virus of mouse pneumonitis. At this dosage level a definite inhibitory effect was obtained against the virus of lymphogranuloma venereum but the activity against the agent of meningopneumonitis was of doubtful significance. By increasing the dose definite effects were obtained against the cat pneumonitis virus with 10 mg per day and against the meningopneumonitis virus with 5 mg.

Similar experiments in mice were done with four derivatives of 5-nitro-2 furaldehyde[§] using daily doses of 2 to 3 mg per mouse, which was close to the maximum amount tolerated over a period of one week. The mortality from toxic effects of the drugs ranged from 10 to 30% of the treated mice in most of the experiments with these substances. From the results presented in Table II, it will be seen that these nitrofurans had definite inhibitory activity against the agents of mouse pneumonitis, cat pneumonitis, lymphogranuloma venereum, and meningopneumonitis. The most active compound under the conditions of these experiments was the semioxamazone, while the diacetate was found to be the least effective. With the former compound at a dosage of 3 mg marked inhibition of the pulmonary lesions produced by the virus of lymphogranuloma, and the meningopneumonitis virus were observed. When the dosage was reduced to 2 mg toxicity of the drug was not evident in the 6-day period of the experiment, but the effect on the pulmonary lesions produced by the meningopneumonitis virus was not as great.

[‡] Kindly furnished by Dr. F. Stimpert, Parke Davis & Company.

[§] 5-nitrofuraldehyde diacetate was purchased from the Eastman Kodak Co. The other three compounds were given by Dr. Mary F. Paul of the Eaton Laboratories, Inc.

TABLE IV.
Effect of Nitrocompounds on Allantoic Infections with Meningopneumonitis Virus in Chick Embryos.

Drugs			Degree of infection as indicated by subinoculation of allantoic fluid into mice			
Name	Dose, mg	Route	LS<20	LS20-50	LS>50	Average L.S.
NF I*	1.0	A	10	12	7	37
Saline		A	2	1	27	83
NF II†	1.0	YS	4	1	1	15
Saline		A	1	2	3	59
Nitroakridin 3582	0.3	A	14	0	0	1.5
Saline		A	1	3	14	74

* NF I = 5-nitro-2-furaldehyde-semioxamazone autoclaved in dry state.
† Filtered through fritted glass (see Tables II and III for name and formula).

Results in chick embryos. Evidence for inhibition of the multiplication of the viruses by nitro-compounds was obtained from experiments in chick embryos. Since the effectiveness of p-nitrobenzoic acid or its amide and p-nitrobenzene sulfonamide seemed to be limited largely to the agent of cat pneumonitis only the experiments in chick embryos with this virus are presented in Table III. Eleven-day-old embryos were infected with a 10^{-3} dilution of yolk sac suspension given by the allantoic route. One hour later the drug was inoculated either by the same route or into the yolk sac. Embryos seldom died as the result of infection but were sacrificed on the 5th day after inoculation and the amount of virus in the allantoic fluid measured by the pulmonary lesion scores resulting from subinoculation intranasally of these fluids into mice which were then sacrificed on the 14th day. As shown in Table III, the allantoic fluids from the majority of the embryos treated with the p-nitrophenyl compounds contained insufficient virus to produce lesion scores of 20 or more in mice while the fluids from many of the control eggs produced lesion scores over 50. The average of the lesion scores from the individual eggs in each group is shown in the last column. The effects were considered significant when the average from the treated eggs was one-half to one-third of the average lesion scores produced by fluids from the control eggs. Chloramphenicol was found to be much more effective against allantoic infections with this virus in chick embryos than it was in mice. The results with sulfanilamide and penicillin are included for comparison. Sulfanilamide at a dosage of 1 mg, comparable to p-nitrobenzene sulfonamide, had no significant effect. The effect produced by a single dose of 0.3 mg (500 units) of penicillin G was equivalent to or only slightly greater than the effect of the nitro-compounds.

The results of similar experiments with nitrofurans and the agent of meningopneumonitis are presented in Table IV. A number of experiments with these compounds sterilized by heating suspensions in saline or starch-saline at 100° for 20 minutes were negative, probably because of heat lability of the drug.[‖] Several experiments were then done with the semioxamazone which was sterilized by heating in the dry state, but the results were not as good as would be expected from the experiments in mice. Since 5-nitro-2 furaldehyde-2-β-hydroxyethyl semicarbazone is soluble in water to the extent of slightly more than 1 mg per cubic centimeter at room temperature, solutions of this substance were filtered and 1 cc inoculated into the yolk sac. In this experiment a definite inhibition of growth of the meningopneumonitis virus in the allantoic sac was observed. In a similar experiment with the cat pneumonitis virus (see Table III) using a dose of 0.5 mg of this same substance no effect was obtained. The results of an experiment with nitroakridin 3582, (3 nitro- 6,7-dimethoxy-9-(2-hydroxy-3-diethyl amino propylamino) acridine, are also included in Table IV. It is obvious that this compound produced a much more marked inhibition than the nitrofurans in the allantoic sac but from previous investi-

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[‖] Mary F. Paul, personal communication.

TABLE V.
Effect of Nitrocompounds on Yolk Sac Infections in Chick Embryos.

Virus	Drug*	Dose	Degree of infection†				
			LS$<$20	LS20-50	LS$>$50	D	Mortality ratio
Mouse pneumonitis	PNB	9 mg	2	2	0	5	5/9
	Saline		3	1	1	8	8/13
Cat pneumonitis	PNB	9 mg	8	0	1	2	2/11
	Saline		5	0	0	10	10/15
	NF II	0.5 mg	7	0	3	0	0/10
	Saline		0	0	2	7	7/9
Meningo-pneumonitis	PNBa	10 mg	0	1	1	4	4/6
	Saline		0	0	4	4	4/8
	NF II	0.5 mg	4	2	0	0	0/6
	Saline		0	1	1	9	9/11

* PNB = p-Nitrobenzoic acid-sodium salt.
PNBa = p-Nitrobenzoic acid-amide.
NF II = 5-nitro-2-furaldehyde-2-β-hydroxyethyl semicarbazone sterilized by filtration.
† In eggs surviving to 8 days. D represents number of embryos dead by 8 days.

gations,[1] it seemed to be less effective than the nitrofurans against the meningopneumonitis virus in the lungs of mice.

The results of experiments in which both drug and virus were inoculated into the yolk sacs of chick embryos are presented in Table V. Sodium p-nitrobenzoate in large doses had no effect on the multiplication of the mouse pneumonitis virus but definite evidence of inhibition of the virus of cat pneumonitis was obtained. In one experiment p-nitrobenzamide had no effect on the agent of meningopneumonitis. The compound 5-nitro-2-furaldehyde-2-β-hydroxyethyl semicarbazone inhibited the growth of both the cat pneumonitis and meningopneumonitis virus in the yolk sac. While most of the control eggs in these two experiments were either dead or heavily infected by the 8th day after inoculation, the majority of the treated eggs survived with insignificant amounts of virus, although a few had moderate to heavy infections. These results with the three viruses and two types of compound parallel quite closely the observations with mice as reported above.

Negative results. The following additional substances containing a nitrophenyl group were screened against the virus of meningopneumonitis in mice and in some cases against other viruses in mice and chick embryos and found to have no effect: p-nitrobenzene sulfonanilide, m-nitrobenzene sulfonamide, sodium p-nitrobenzene sulfonate, p-nitroacetanilide, 3-nitro-4-acet-toluide, N(5-nitro-2-furylidene)-1-aminohydantoin.

Discussion. These observations and the results reported by other investigators suggest that a certain chemical structure containing a nitro substituent confers on several substances activity against viruses of the psittacosis-lymphogranuloma group. The only chemical grouping common to most of the active nitro compounds so far found is the ring-system of carbon atoms with conjugated double bonds thru which the nitro group is joined to various chemical complexes of rather wide diversity.

It is likely that the nature of the substituent on the benzene ring in the para position to the nitro group plays an important part in determining chemotherapeutic and pharmacologic properties. An analogy with the sulfonamides exists in that the substituent on the sulfonic acid group of the latter compounds increases or decreases their activity. Although some of the compounds listed in the present work resemble p-aminobenzoic acid, others such as the nitrofurans have a structure which is quite different from PABA. Since PABA itself has been found to be inactive against agents of the psittacosis-lymphogranuloma group[6,7] the effects of p-nitrobenzoic acid and its derivatives cannot be attributed to reduction of the NO_2 group to NH_2 *in vivo*. The possible effect of this reduction in other compounds has not to our knowledge been investigated.

From a consideration of the spectrum of antiviral activity of these substances it is evident that the nitro-compounds as a group, including nitroacridines, have a somewhat similar range of activity while differences from sulfonamides may be found. The agent of mouse pneumonitis which is highly sensitive to sulfonamides, has a relatively low sensitivity to nitroacridine[1] and is not inhibited significantly by derivatives of p-nitrobenzene with the exception of chloramphenicol. The cat pneumonitis and meningopneumonitis viruses are resistant to sulfonamides but sensitive to the action of nitroacridines and to most of the other nitro compounds in the case of the former virus but only to nitrofurans and chloramphenicol in the case of meningopneumonitis.

Under the conditions of these experiments the nitrofurans seemed to have a wider range of activity against viral respiratory infections in mice than did the other nitro compounds. The results with chloramphenicol are in agreement with the report of Smadel and Jackson[8] who demonstrated activity of this substance against the agents of lymphogranuloma venereum and psittacosis in chick embryos and against psittacosis in mice inoculated by the intraperitoneal route but were unable to find significant activity against these viruses in mice inoculated by the intracerebral route. From the results presented in Tables I and II it may be seen that chloramphenicol seemed to be less active against the agent of cat pneumonitis than were some of the other nitro compounds. On the other hand, chloramphenicol was more active against the agents of lymphogranuloma venereum and meningopneumonitis than were any of the other nitrophenyl compounds, but was somewhat less active than the nitrofurans.

While the observations reported here reveal interesting relationships between chemical structure and activity against the viruses of the psittacosis-lymphogranuloma group, it is doubtful that the chemotherapeutic index of any of the substances tested is favorable enough to justify human use. Although sodium p-nitrobenzoate is of low toxicity in mice it has a relatively low activity which is limited almost entirely to the agent of feline pneumonitis. Nitrosulfonamides have been used in human beings for other purposes,[9] but their activity against the psittacosis-lymphogranuloma group is also limited. The nitrofurans have a relatively high degree of activity against these viruses but they have a definite toxic action and have been shown to produce delayed lethal effects in guinea pigs several days after discontinuance of administration.[10] Chloramphenicol is probably among the least toxic of the substances tested but its activity against the agents of the psittacosis-lymphogranuloma group does no seem to be greater than that of penicillin or the sulfonamides.[8]

Summary. Several substituted nitrobenzenes including chloramphenicol (chloromycetin) show inhibitory activity against the viruses of meningopneumonitis, lymphogranuloma venereum, cat pneumonitis, and mouse pneumonitis. Several nitrofurans were also shown to inhibit the growth of these viruses. The chemotherapeutic activity of these various substances is compared and the relation of chemical constitution to activity discussed. Although chloramphenicol is probably the least toxic of the substances tested, it did not show striking superiority to other nitro-compounds in activity against viruses of the psittacosis-lymphogranuloma group under the conditions of these experiments.

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[7] Hamilton, H. L., PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 220.

[8] Smadel, J. E., and Jackson, E. B., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 478.

[9] Major, R. H., *J. Lab. and Clin. Med.*, 1946, **31**, 219.

[10] Wolinsky, E., Wetzel, V., and Steenken, W. J., PROC. SOC. EXP. BIOL. AND MED., 1949, **70**, 483.

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17239. Inhibition of Agents of the Psittacosis-Lymphogranuloma Group by P-Arsenobenzamide.

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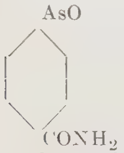
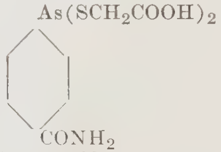
Recent investigations of nitro-analogues of para amino benzoic acid have revealed activity of certain of these compounds against agents of the psittacosis-lymphogranuloma group.[1] Because of these observations it seemed of interest to test compounds recently available in which arsenic is substituted for the nitrogen atom and p-arseno benzamide was selected because it may be considered an arsenic analogue of p-nitro benzamide. The latter substance has shown slight inhibitory activity against the virus of feline pneumonitis. p-Arsenobenzamide and the dithioglycollate, known as Arsenamide,* were tested in mice and chick embryos by the methods described in a current publication[1] against the agents of meningopneumonitis, cat pneumonitis and mouse pneumonitis.


I p-arsenobenzamide


II Arsenamide

The results of experiments on pneumonia in mice are summarized in Table I. In mice inoculated intranasally with the agents of mouse or cat pneumonitis and treated with daily intraperitoneal doses of 0.4 mg of p-arsenobenzamide the gross pulmonary lesions were reduced by about 50 per cent as compared with the controls, and there was also significant reduction in consolidation as measured by the lung weights. The thioglycollate derivative, Arsenamide, was about equally effective against the agent of cat pneumonitis.

Chick embryos infected by the allantoic route were given a single dose of 0.2 mg of Arsenamide by the same route after the virus. The results are summarized in Table II. When the substance was given one hour after inoculation of the virus almost complete inhibition of the growth of the agent of cat pneumonitis was observed, as determined by subinoculation of the allantoic fluids of individual eggs into mice. At 24 and 48 hours after inoculation Arsenamide produced partial inhibition of the growth of this virus. The results with meningopneumonitis virus were less striking but partial inhibition of growth in the allantoic sac was obtained when the drug was given one hour after the virus. In mice no significant effect against this virus was obtained (Table I).

Comment. When tested under the same experimental conditions, the activity of p-arsenobenzamide and Arsenamide compares favorably on a weight basis with some of the nitro-compounds including chloromycetin but their toxicity is relatively high and further investigation may show their range of activity to be limited.

As other arsenic compounds have not been examined these preliminary results do not establish any relation of structure to activity against the psittacosis - lymphogranuloma group and further studies will be necessary to assess the significance of the structural relation of this compound to p-aminobenzoic acid and p-nitrobenzamide. Williamson and Lourie[2] have reported that PABA interferes with the trypanocidal action of γ (p-arsenophenyl)-butyric acid, a compound of somewhat similar structure, and Schleyer and Schnitzer[3] find that esters and amides of substituted benzoic acids, but not the acids themselves, antagonize the effect of mapharsen and acriflavine.

Summary. p-Arsenobenzamide and its

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[1] Eaton, M. D., Huang, C., and Levenson, C. G., Proc. Soc. Exp. Biol. and Med., 1949, **71**, 501.

* Both supplied by Dr. Marlin T. Leffler of the Abbott Laboratories.

[2] Williamson, J., and Lourie, E. M., *Ann. Trop. Med. and Parasit.*, 1947, **41**, 278.

[3] Schleyer, W. L., and Schnitzer, R. J., *J. Immunol.*, 1948, **60**, 265.

TABLE I.
Effect of p-Arsenobenzamide and its Dithioglycollate on Viral Respiratory Infections in Mice.

Virus	Compound	Daily dose, mg	No. of mice	Lesion score		Lung weight	
				T/C*	% change†	T/C*	% change†
Cat pn.	I	0.4	27	21/40	—47	0.203/0.311	—35
” ”	II	0.5	32	22/39	—44	0.266/0.311	—15
Mouse pn.	I	0.4	27	11/25	—56	0.161/0.209	—23
Meningo-pneum.	I	0.2	16	30/35	—15	0.297/0.325	— 8

* T = Treated mice. C = Control mice.
† % change = 100(—1+T/C).

TABLE II.
Effect of Arsenamide on Allantoic Infections in Chick Embryos.

Virus	Drug* time After virus, hr	Degree of infection as indicated by subinoculation of allantoic fluids into mice†			
		LS<20	LS20-50	LS>50	Av. L.S.
Cat pneumonitis	1	12	0	0	3.0
	Sal.	2	5	7	52.0
	24	7	5	0	16.3
	48	15	1	1	15.2
	Sal.	3	1	4	46.0
Meningo-pneumonitis	1	10	5	2	22.0
	Sal.	0	1	11	87.0

* Dose 0.2 mg/egg by allantoic route.
† LS<20, allantoic fluid not significantly infected; LS20-50, moderate infection; LS>50, heavily infected allantoic fluid.

dithioglycollate have a definite inhibitory activity against the viruses of mouse pneumonitis and cat pneumonitis in the lungs of mice. The dithioglycollate also inhibits growth of the agents of meningopneumonitis and cat pneumonitis in the allantoic sac of chick embryos.

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17240. Vitamin B_{12} Content of Various Organs and Tissues of the Rat.*

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The glandular tissues of various domestic animals have long been known to be effective in pernicious anemia when administered orally.[1] Beef liver and kidney have proved to be the richest source of the active principle, kidney, however, possessing only one-half to two-

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We are indebted to Merck and Company, Inc., Rahway, N. J., for crystalline vitamins and the crude B_{12} concentrate, and to Dr. B. L. Hutchings of the Lederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y., for synthetic folic acid.

[1] Subbarow, Y., Hastings, A. B., and Elkin, M., *Vitamins and Hormones*, 1945, **3**, 237.

TABLE I.
Vitamin B_{12} Content of Rat Tissues.

Daily supplement	No. of animals per assay group	Series 1 Total avg gain (g) 2 wk	Series 2 Total avg gain (g) 2 wk	Min. B_{12} (γ) per g of sample
None	5	48	37	—
.1 γ crystalline B_{12}	5	73	66	
1 g kidney	3	65	51	0.04
1 g '' (B_{12})*	3	89	87	0.17
1 g liver	5	47	31	†
1 g '' (B_{12})	5	75	66	0.08
1 g intestine	5	38	—	†
1 g '' (B_{12})	5	62	—	0.04
1 g heart	1	—	36	†
1 g '' (B_{12})	1	—	62	0.07
1 g spleen	1	—	32	†
1 g '' (B_{12})	1	—	30	†
2.5 g muscle	5	57	43	†
2.5 g '' (B_{12})	5	71	66	Trace

* (B_{12})From rats that received B_{12} concentrate.
† No measurable quantity.

thirds the activity of liver. Spleen, brain, heart, and pancreas are effective to a lesser extent. These same organs from the pig and lamb have likewise been used beneficially in the dietary treatment of pernicious anemia.

An assay method has been reported[2] which can be used to measure fairly quantitatively the activity of anti-pernicious anemia preparations, and all subsequent work with the assay has indicated that vitamin B_{12} is the active component being determined. The quantitative response obtained with crystalline vitamin B_{12} affords a basis for the estimation of the amount of B_{12} present in the material tested. Application of this was made in the testing of various beef and pork samples for B_{12} content.[3] The work to be reported was undertaken to determine the relative concentration of vitamin B_{12} in various organs and tissues of the rat in an effort to locate the main storage site of the vitamin within the body.

Experimental. Animals. The rats used as sources of the organ and tissue samples were obtained as weanlings. They were housed in stock colony cages and given food and water *ad libitum*. A total of 30 rats were used and all received the corn-soybean ration described previously.[4] Fifteen of the animals were given in addition to this diet a vitamin B_{12} concentrate (Merck and Co.) which was mixed directly into the basal ration to provide 3 γ of B_{12} per 100 g of food. The rats were kept on these diets for 6 weeks before sacrificing.

Preparation of samples. The rats were decapitated, bled, and the tissues and organs removed. The liver, kidney, heart, spleen, small intestine, and the femoral muscles were taken. The livers, kidneys, and intestines were weighed, combined with an equal weight of distilled water, and homogenized in a Waring blendor The skeletal muscle was treated in the same manner except that a 3 to 1 dilution was necessary to obtain a smooth homogenate. All samples were stored at ordinary refrigeration temperature.

Assay. The assay method used has been described in a previous paper.[2] It consists of placing weanling rats on a basal ration for a 2-week depletion period and following the growth response during another 2-week period when the material to be tested is given. The samples were administered on alternate days in separate food containers. Four ml of the liver and kidney homogenates were given every other day, thus supplying the animals with an equivalent of 1 g of the sample per day. Because of the higher dilution of the muscle homogenate and its probable lower B_{12} content, 15 ml were administered which provided 2.5 g of muscle tissue per day.

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[2] Register, U. D., Ruegamer, W. R., and Elvehjem, C. A., *J. Biol. Chem.*, 1949, **177**, 129.

[3] Register, U. D., Lewis, U. J., Thompson, H. T., and Elvehjem, C. A., PROC. SOC. EXP. BIOL. AND MED., 1949, **70**, 167.

[4] Jaffé, W. G., and Elvehjem, C. A., *J. Biol. Chem.*, 1947, **169**, 287.

The number of rats used for each assay group is recorded in Table I. A smaller number of rats was necessary in the cases of the kidney, spleen and heart because of an insufficient amount of sample. The assay was run in two series, a different group of rats being used as a source of the organs and tissues in each case.

Results and discussion. The data on the vitamin B_{12} content of all the rat tissues and organs tested are presented in Table I. The values obtained for the heart and spleen are based on results from one assay animal and, therefore, are to be regarded only as indications of the actual.

The greater weight gain in the first series can be explained by the fact that the basal gain, that is, without supplement, was higher throughout all the groups. However, the relative values for B_{12} content of the samples in the two individual series were similar.

The data show that in all instances, except possibly with the spleen, the inclusion of the B_{12} concentrate in the animal's diet caused an increase in the quantity of B_{12} in the organs and tissues.

The kidneys were found to be the site of greatest B_{12} concentration. Also the results indicate that the vitamin is retained in larger quantity for a longer period of time in the kidneys than in the other organs. The liver, heart, and intestine contained no appreciable amount of vitamin B_{12} after the animals had been kept on the corn-soybean ration for 6 weeks. However, the increase was very marked in these organs, as with the kidney, upon the addition of the B_{12} concentrate to the rat's diet. The skeletal muscle was not only lowest in B_{12} but also remained most nearly constant in content.

Summary. The kidney, liver, heart, small intestine and femoral muscles of rats raised on a corn-soybean diet were assayed for vitamin B_{12} content.

The kidney was found to be the site of greatest B_{12} concentration. The liver, heart, small intestine, and muscle contained no appreciable amount of the vitamin after the animals had been kept on the basal ration for 6 weeks. The amount of B_{12} increased in the organs and tissues when vitamin B_{12} was added to the diet.

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